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THE RESPIRATION OF *TYROGLYPHUS FARINAE*

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(With Two Text-figures)

This investigation into the reactions of *Tyroglyphus farinae* to varying concentrations of carbon dioxide and oxygen and to the presence of small quantities of hydrocyanic acid, was carried out in order to obtain some insight into the respiratory activities of the mite, very little being known of its physiological reactions. As a necessary preliminary, its reactions to humidity were determined. *T. farinae* belongs to the family Tyroglyphidae and respires by direct diffusion through the body surface, since no tracheae are present. These mites infest stored food material, being found on grain, flour and meal. They are associated with the micro-organisms of decay and it might be expected that the gaseous conditions under which they live differ considerably from those of ordinary air. It was decided, therefore, to isolate cultures and to investigate the gaseous changes produced by the organisms in the culture and the effect of such changes on the mites present. The respiratory quotient in normal air was also determined, together with the quotient obtained in the presence of an $M/1000$ solution of potassium cyanide. The reactions of the mite to varying concentrations of carbon dioxide, oxygen and water vapour in the atmosphere were also determined.

A. HUMIDITIES

Small quantities of culture were placed in wide specimen tubes, the mouths of which were covered with fine silk material to enclose the animals. These tubes were placed in desiccators containing sulphuric acid of known specific gravity, and the desiccators kept in an incubator at 20° C. Five tubes were used in each case. At intervals, the tubes were removed and examined for live mites under a low-power binocular microscope. Mites cultured in flour and on whole grain were used.

Table 1

Time after	R.H. %						
	20	45·5	58·3	65·5	74	80	90
1 day	D	A	A	A	A	A	A
2 days	.	D	A	A	A	A	A
3 days	.	.	A	A	A	A	A
4 days	.	.	D	A	A	A	A
5 days	.	.	.	A	A	A+	A+
6 days	.	.	.	A	A	A+	A+
7 days	.	.	.	A	A	A+	A+
8 days	.	.	.	A	A	A+	A+
9 days	.	.	.	A	A+	A+	A+

Abbreviations: A = alive, D = dead, A+ = alive and breeding.

The lower limiting factor of humidity was found to lie between R.H. 58·3% and R.H. 65·5% at 20° C. This result appears to be in accordance with the work of A. M. Hora (1933) on *Glycyphagus domesticus* de Geer, where a lower limit of R.H. 70% at 25° C. was obtained. It is difficult to obtain results at humidities above 90% owing to condensation which tends to drown the mites when tubes are removed for inspection.

B. GASEOUS CHANGES PRODUCED BY ENCLOSING THE CULTURES

A strong culture of mites in flour was placed in a conical flask (in this case about an ounce of culture was used) which was joined to another by a wide-bore tube. Into the second flask, sulphuric acid of sp. gr. 1·2 could be run from a tap funnel; a delivery tube led from the first flask into a beaker of glycerine. The two flasks stood in a water-bath at 25° C. A certain amount of carbon dioxide would be lost by solution in the acid, and, owing to the delivery tube being in glycerine, there would be a humidity gradient from the flask containing acid to the surface of the glycerine in the tube. Even so, the humidity in the flask remained sufficiently high to prevent death by desiccation, as would have happened if glycerine had been used in the place of the sulphuric acid. At intervals, 20 c.c. of air were displaced, collected over glycerine to prevent further loss of carbon dioxide, so that the actual concentration above the culture might be known. This air was collected in a small eudiometer tube, transferred to the clamp of a levelling apparatus, so that all volumes could be read with the levels inside and outside the tube equal. The tube having been levelled in a tall jar of glycerine, the volume was read. To absorb carbon dioxide, large drops of 20% potassium hydroxide solution were injected up the tube by means of a bent pipette of 5 c.c. capacity. Three consistent readings were obtained before a 5% solution of pyrogallic acid in 5% potassium hydroxide was added to absorb the oxygen. The temperature was noted at each reading, and any necessary correction having been made, the percentages of carbon dioxide and oxygen present were calculated and plotted on a graph of gas concentration against time. The respiratory quotient for the culture as a whole was calculated, and is seen to drop as the quantity of carbon dioxide increases. The graph reproduced shows the type of changes which took place in the atmosphere over the flour in this series of experiments.

Table 2

Time days	CO_2 %	O_2 %	R.Q.
8	3·553	17·26	0·95
20	6·77	13·56	0·909
27	7·263	11·9	0·798
32	8·872	6·403	0·607
36	9·754	6·340	0·66
44	8·437	6·21	0·57
50	9·001	5·00	0·56

The carbon dioxide concentration rose and became steady between 9·75 and 10·5%, the oxygen content having dropped to 6·3–5·00%. On opening the cultures, the mites were found to be anaesthetized. If the cultures were given access to air, the mites recovered in a few hours.

Newstead and Morris (1920) exposed *T. farinae* to pure carbon dioxide and found that the mite was anaesthetized but not killed. Few times of exposure, however, are given. The anaesthetic effect in isolated cultures might either be due to the increased carbon dioxide concentration or the low oxygen concentration. On comparison with the results of (E) it became evident that the anaesthetic effect was probably due to accumulation of carbon dioxide since the mites are tolerant of low concentrations of oxygen.

The respiratory quotient for the enclosed cultures was found to be in the neighbourhood of 0·9 at first, which is in agreement with the R.Q. for mites in the absence of culture medium, as determined in the series of experiments (C). In point of fact, a R.Q. of this order is only what one would expect for animals living in a medium very rich in carbohydrate which must form the principal respiratory base.

The R.Q. of isolated cultures begins to drop as the carbon dioxide concentration above the culture rises; this may be due to increased loss of carbon dioxide in the sulphuric acid used to regulate the humidity and to displace the air samples for analysis, or it may be due to the mites beginning to oxidize a fat as anaesthesia approaches.

C. RESPIRATORY QUOTIENT IN ORDINARY AIR

Thirty to forty mites were enclosed in a hypodermic syringe, the open end of the needle being immersed in glycerine. After intervals of 6, 12 and 24 hr. a small bubble of air was injected into a Krogh micro-analysis apparatus as modified by Campbell & Taylor (1935). This air bubble was analysed for carbon dioxide and oxygen and the respiratory quotient calculated.

Table 3. *Respiratory quotient over air at laboratory temperatures of* 10–17° C.

0·8830	0·8900	1·2900	0·8700
1·1700	0·9212	1·1600	1·1670
0·7264	0·9363	0·7457	0·7800
0·7300	0·9500	0·8000	0·9500
0·9520	0·8900	0·9170	0·9450
1·1300	0·8300	0·8900	

Total 21·5236

Variance = 0·02272
S.D. $\sqrt{v}$ = 0·1508
S.E. S.D./$\sqrt{n}$ = 0·03144
Mean = 0·9358

The mean R.Q. 0·9358 with a standard error of 0·03144 would indicate a normal process of aerobic respiration of a carbohydrate base. This R.Q. is in close agreement with that obtained in the early stages of experiment (B). The mites do not show any alteration in R.Q. due to lack of food in intervals up to 24 hr., since all the R.Q.'s obtained showed the same sort of variation whether after 6, 12 or 24 hr.

D. THE RESPIRATORY QUOTIENT IN THE PRESENCE OF POTASSIUM CYANIDE

The same apparatus was used for investigating the effect of hydrocyanic acid, a drop of *M*/1000 potassium cyanide solution being suspended on the plunger.

Table 4

No. of mites	R.Q. after 12 hr.	R.Q. after 24 hr.
40	1·69	0·86
40	1·62	0·88
35	1·48	0·85
35	1·28	0·93
40	1·72	0·93
40	1·8	0·85
35	1·2	1·16
30	1·2	1·17
30	1·077	1·07
30	1·1	Mean 0·966
30	1·1	Variance 0·0154
30	1·04	S.D. 0·1241
35	1·12	S.E. 0·0415
30	1·14	
30	1·14	
30	1·14	
30	1·1	
40	1·37	
40	1·6	
	Mean 1·323	

The mean quotient obtained at the end of 12 hr. was 1·323. The actual quotient obtained in any experiment depends apparently on the number of mites used. No results below unity were obtained. From this it seems evident that the absorption of oxygen is largely cyanide-sensitive, and that the animals respire by means of a cytochrome and cytochrome oxidase which is sensitive to cyanide (Keilin, 1929), as is the normal case in aerobically respiring organisms.

The return after 24 hr. to a R.Q. which shows close agreement with that obtained in ordinary air, indicates that, with this degree of inhibition of the oxidation of reduced cytochrome, the animals survive long enough for the production of fresh cytochrome oxidase, or else destroy the absorbed cyanide by some process of tissue metabolism.

E. REACTIONS OF *T. FARINAE* TO LOW CONCENTRATION OF OXYGEN

Tubes were set up as for the humidity experiments and stood over alkaline pyrogallol solution in glass-stoppered jars. The stoppers were well greased and a seal of heavy engine oil was run into the grooves between the stoppers and the top edges of the jars. The bottles were opened one at a time and the tubes examined for live mites. Cultures on whole grain and in flour were used. In the case of the flour, ¼ in. of flour in a 2½ × ¾ in. specimen tube was used.

The tubes over pyrogallol were assumed to be in an atmosphere of low oxygen concentration and saturated with aqueous vapour. After 24 hr. the mites were alive and feeding; after 36 hr. a number were dead; after 48 hr. there were still a few alive. After 72 hr. all were dead in the case of cultures in flour.

Table 5

Time hr.	State of culture in flour	State of culture on grain
12	Alive and feeding	Alive but still
24	Alive and feeding	Alive but still
36	Most alive, a few dead	Alive but still
48	Majority still, a few sluggish	Majority dead
60	Majority alive but still	Dead
72	All dead	

The fact that *T. farinae* can in some cases, at any rate, survive 48 hr. in an atmosphere of low oxygen concentration, would at first sight seem to indicate that it is capable of anaerobic respiration for a limited length of time. This is in accordance with the results obtained in (D). It also seems to confirm the view that inactivity of mites in isolated cultures is due to the accumulation of carbon dioxide rather than the diminution in oxygen. However, a comparison of the results obtained when using mites cultured in flour or on whole grain and with the results of (F) shows conclusively that the activity of mites in tubes of flour over pyrogallol is due to the slowness with which an equilibrium is set up, and not to the ability of the mites to respire anaerobically to such an extent as to remain active.

The time lag of the atmosphere in flour in coming to an equilibrium with the outside atmosphere is seen when the results of (E) and (F) are compared; mites remaining active in flour stood over pyrogallol for at least 36 hr.; whereas the effect of atmospheric nitrogen on exposed mites is to produce an almost instantaneous anaesthesia. For this reason, it is important to use either mites picked out on a needle or else a heavily infected grain when determining the R.Q. or the relation to humidity.

F. REACTIONS TO VARYING CONCENTRATIONS OF CARBON DIOXIDE AND OXYGEN IN AIR

Mites were placed in an observation tube and air of known composition as regards to carbon dioxide and oxygen run through it. The air was mixed in the apparatus shown in Fig. 1. This apparatus was used as follows: The gas chamber was filled with glycerine and by means of the levelling cup, known quantities of air, carbon dioxide and atmospheric nitrogen were drawn in. The gases drawn in were washed by being aspirated through water. The atmosphere thus mixed could then be displaced by means of the levelling cup into the tube containing the mites. Since the volume of air passed through the observation tube was many times its own volume, it was assumed that the

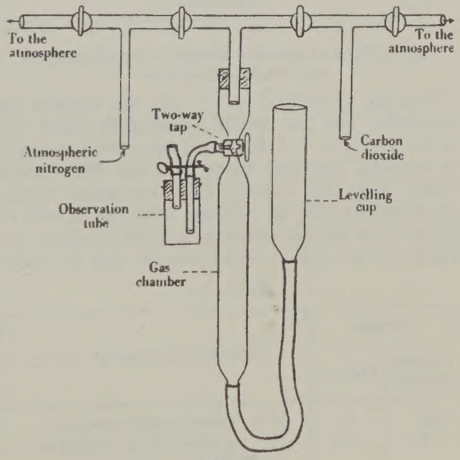

Fig. 1

final atmosphere would be the same as that mixed in the apparatus. The observation tube was then sealed with a spring clip and put into a water-bath at 20° C. At intervals the mites were observed under the microscope. To avoid error in mixing the atmosphere, care was taken to pass plenty of carbon dioxide and nitrogen through the two-way tap before starting the actual mixing. Carbon dioxide was generated by the action of sulphuric acid on sodium carbonate and passed through two wash bottles of distilled water before passing into the apparatus. Atmospheric nitrogen was prepared by using a Winchester quart with 100 c.c. of alkaline pryogallol in it; this was shaken up repeatedly and the bottle allowed to stand for 24 hr. The gas was displaced into the apparatus by running distilled water into the Winchester from a large tap funnel. Care was taken to end all measurements of volume with the levels of glycerine in the gas chamber and the levelling cup equal. If the experiments were to last for more than 24 hr. a fragment of a grain was placed in the observation tube to supply food, and to prevent alterations due to respiration accumulating, the atmosphere was renewed every 24 hr.

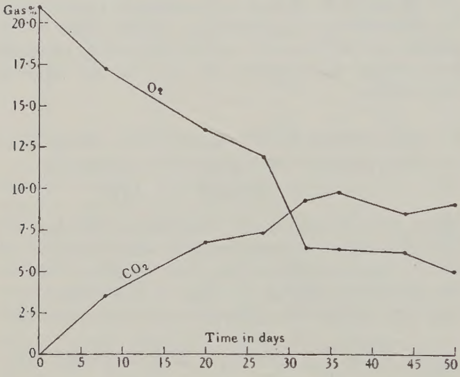

Fig. 2. Type of gaseous change in the atmosphere over enclosed cultures

Table 6

Dosage		Conditions within an hour
Carbon dioxide	Oxygen	
100	0	M –, almost instantaneously
37·5	12·5	N – –, more often, complete immobility
30	14	N – –, more often, complete immobility
25	15	N –, majority still
20	16	N –, differing very little from normal
15	17	N
0	2·4	N, possibly a little sluggish
0	1·6	N –
0	1·2	N – –, sometimes N –
0	0·8	M, sometimes N – –
0	0·4	M, immediately

Table 7

Gas concentration		Time and condition after dosage				
CO_2	O_2	12 hr.	1 day	2 days	3 days	4 days
100	0	M	M	M	M	M*
27·5	14·5	N – –	M	M	M	M
20	15	N –	M	M	M	M
20	16	N	N	N	N	N
0	2·0	N	N	N	N	N
0	1·6	N –	N –	N –	N –	N –
0	1·2	N –	N –	N –	N – –	N –
0	0·8	N – –	N – –	M	M	M
0	0·4	M	M	M	M	M
0	0	M	M	M	M	M*
10	5	N	N	N	N	N

* Dead after 72 hr.

Abbreviations used in Tables 6 and 7: N denotes normal movement of mites; N –, sluggish walking movement; N – –, feeble twitching of limbs; M indicates immobility.

These experiments were repeated upwards of 20 times with consistent results.

From the results of this series of experiments it is seen that the value of carbon dioxide as a factor limiting activity is in the neighbourhood of 30%. Concentrations above this produce an almost instantaneous anaesthesia. For oxygen this value lies between 1·2 and 0·8%.

It is immediately obvious that these figures are at variance with those obtained in series (B), where the culture stabilized with an external atmosphere of 10% carbon dioxide and 5% oxygen. I am of the opinion that the micro-atmosphere inside the flour had, when these external conditions had been reached, an entirely different constitution. The concentration of carbon dioxide must have been much higher than 10% or the concentration of oxygen much lower than 5% to produce anaesthesia and eventually death. The fact that flour which has stood over pyrogallol—an active absorbent of oxygen—contained active mites after 36 hr., whereas pure nitrogen has an instantaneous effect, indicates the slowness with which the micro-atmosphere is affected by external conditions; so that it is not really surprising that the atmosphere above an isolated culture should be very slow to show the increase in carbon dioxide and the diminution in oxygen which must be going on inside it.

The results obtained in the series (F) indicate that these mites have a very high resistance to carbon dioxide accumulation and to lack of oxygen. This would be an obvious adaptation to the conditions under which they live. The ccumulation of carbon dioxide and lack of ygen inside large masses of grain and flour must very important factors in the physiological ocesses of the organisms living in them. The w concentration of the oxygen at which mites main active would indicate the presence of an ygen carrier in the fluid of the vacuolated cking tissue of the body. This activity at low ygen concentration is met with in some insect rvae where it would seem to be correlated with e presence of haemoglobin. No such pigmented rrier is visible in *T. farinae* (Harnisch, 1937).

CONCLUSIONS

1. *Tyroglyphus farinae* is not viable at R.H. of s than 65·5 % at 20° C.

2. The normal R.Q. is in the neighbourhood of 0·9, indicating respiration of a carbohydrate base.

3. The oxygen absorption is in part cyanide-sensitive, which suggests the presence of a 'cytochrome'-'cytochrome oxidase' mechanism.

4. Anaesthesia can be produced by concentrations of carbon dioxide above 30 % of the atmosphere and by low oxygen concentrations below 1·2 %.

5. The external atmosphere over flour is no guide to the internal conditions within the flour, even when both are enclosed.

6. If exposed to pure carbon dioxide for 72 hr. the mites are killed.

7. The mites will survive 48 but not 72 hr. exposure to atmospheric nitrogen.

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THE STIMULUS TO FEEDING IN *ANEMONIA SULCATA*

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THE FEEDING RESPONSE

Like all sea anemones, *Anemonia sulcata* possesses a well-marked feeding reaction. A lump of meat placed on the tentacles is instantly seized and rapidly conveyed to the mouth which then ingests it. It is our object to determine the stimuli which cause this response.

The feeding reaction is complex and orderly. There is first a discharge of the cnidae from the tentacles into the food. This discharge is apparently independent of the nervous system (Pantin, 1942), though the increased mechanical contact caused by it helps to stimulate the tentacles. Immediately after contact, the tentacles clasp the food and bend strongly towards the mouth. The edge of the disk carrying these tentacles contracts, so that they bunch together round the food, and then rises up and turns inwards thereby folding tentacles and food towards the mouth. After a few seconds, the part of the oral disk between the food and the mouth slowly contracts and sinks downwards, so that the mouth turns towards the food. The mouth then protrudes and begins to open, the food is gradually thrust in, and muscular action of the pharynx pulls it into the gastral cavity. An essentially similar feeding reaction is found in other anemones.

Normally, a mechanical stimulus alone fails to produce a feeding response. A piece of clean cotton-wool soaked in sea water is handled for a little while by the tentacles as at the beginning of feeding, but these soon fall back and let the wool roll off the disk. However, this rejection is simply an unfinished feeding reaction which fails in its initial stages, and all gradations can be found between the two. As many investigators (Nagel, 1892; Allabach, 1905; Jennings, 1905; Parker, 1917) have shown in other actinians, the response varies greatly with the individual. Starved anemones in good condition may give a complete feeding reaction even to inert objects. After such anemones are given food, the reactions of the mouth and disk and finally of the tentacles themselves towards inert objects become progressively reduced till the feeding passes into rejection. Later, the reaction becomes so depressed that well-fed anemones will even reject food.

A piece of food is always more effective than an inert object. This is due to a chemical stimulus. If about 2 ml. of an active food solution, such as 10 % human saliva in sea water (0·1 % dry weight), is allowed slowly to flow over one group of tentacles of *Anemonia*, a fairly complete feeding response follows, except that there is no material body for the open mouth to ingest. While therefore both local mechanical and chemical stimuli are together involved in normal feeding, the reaction can on occasion be brought about by localized stimuli that are either purely mechanical or purely chemical. Even local electrical excitation of the tentacles will induce the reaction, so that it is not fundamentally dependent upon the mode of excitation.

The failure of inert objects to cause feeding appears to be due to rapid sensory adaptation to mechanical stimuli. Isolated tentacles touched with a glass rod soon cease to respond, whereas if they are immersed in a food solution, they continue giving contractions for many minutes. This interpretation is borne out by experiments on *Calliactis parasitica* and *Anemonia*. If the tentacles of these anemones are locally stimulated electrically at a frequency of about 1 shock in 4 sec. with non-polarizable electrodes, a series of 3 or 4 shocks causes a typical rejection reaction. If the series of shocks continues, more and more of the feeding reaction is elicited, and after 10 or 20 stimuli movements of the mouth and disk may even lead to ingestion of the electrodes.

The above observations show that it does not matter whether the stimulus is mechanical, chemical or even electrical, but the excitation set up must continue for sufficient time if it is to result in a feeding reaction. Chemical stimuli by food usually result in the necessary prolonged excitation: mechanical stimuli, though sometimes effective, do not usually have this result.

Isolated tentacles and mouths go through their proper reactions in the presence of food. But co-ordination of the feeding response is not simply due to successive localized excitation of autonomous effectors. Electric stimulation of the tentacles gradually causes the mouth to bend towards the stimulated region and to open. In the same way, food placed on the tentacles in *Metridium* will induce the mouth to open even if it is removed before it touches the mouth (Parker, 1917). The mouth can therefore be caused to open by transmission of excitation from the tentacles. Nevertheless, direct chemical stimulation of the mouth by food substances causes it to open and, f there is a solid food object, to ingest. The mouth is therefore also directly excitable chemi-ally. Both indirect excitation from the tentacles nd direct chemical excitation occur in the normal eeding reaction.

According to Nagel (1892, 1894), feeding in nemones is initiated by chemical stimulation of he tentacles. But Loeb (1895) showed that even fter the tentacles were cut off, selective feeding ould still take place through chemical excitation f the mouth. Fleure & Walton (1906) agreed with Loeb and suggested that with natural food he tentacles of *Anemonia* only respond to mechanical stimuli. It is true that the mouth of *nemonia* is sensitive to chemical stimuli and that he tentacles are very sensitive to mechanical timuli, but we have found in all normal specimens f *Anemonia* a clear distinction between the ejection by the tentacles of inert objects like plain otton-wool and their acceptance of solid food or f cotton-wool soaked in food solution. While hemical stimuli to the mouth are important, it those to the tentacles that initiate the feeding eaction. What is this chemical stimulus?

METHODS OF STUDYING CHEMICAL SENSITIVITY

he response of *Anemonia* to food substances and eir derivatives was tested by placing on the ntacles pieces of cotton-wool about 2 ml. in olume soaked with known concentrations of lutions or suspensions of the food substance in a water. Controls were made with wads of otton-wool soaked in sea water or in a standard od solution. In some experiments, the food lution was directly squirted on to the tentacles mouth without cotton-wool.

Among freshly collected animals starved for hr., the threshold concentration of food lution varied as much as tenfold. These animals ere used for trials. Those that were in poor ndition or that had been recently fed required normally high concentrations or even failed to spond to the most potent foods. Such animals ere rejected. On the other hand, exceptional dividuals responded to concentrations three or ur times less than the average; but such animals ight give some feeding response even to inert jects such as plain cotton-wool. Usually six emones were taken and tested simultaneously each trial. Frequent testing of the same emone at short intervals was avoided because pid successive trials of a food substance in e same anemone caused the feeding reaction to ll off owing to adaptation.

Though the sensitivity of individual animals varies considerably, their relative sensitivity to different foods remains very constant. In the experiments extract of minced beef, human saliva, egg white, and Witte's peptone were used as standards by which to compare other substances. Most of the anemones just reacted definitely to pieces of cotton-wool soaked in 0·01 % dry weight of saliva, 1·0 % dry weight egg white or between 1·0 and 0·1 % dry weight Witte's peptone. In the tables given below, the limiting concentrations of other food substances relative to these are given. The limiting concentrations for animals with sensitivity somewhat above or below the average have been scaled down or up according to the values given for the standard substances. Except where otherwise stated, all solid substances were ground up to as fine a state of division as possible while fresh and suspended in sea water. Coarse particles that could not be dispersed were removed. Dry weights were determined for the fresh foods by drying in vacuo to constant weight in a desiccator.

THE RESPONSE TO FOODSTUFFS

With active food substances, contact with the food object is accompanied by strong clinging of the tentacles to it owing to discharge of cnidae. The part played by muscular activity of the tentacles and by the cnidae is not relatively the same for all foodstuffs. Table 1 shows the limiting concentration expressed as dry weight which will cause the tentacles to initiate a feeding response to cotton wads soaked in a variety of food substances. When all allowance has been made for individual variability, it is still evident that food substances differ enormously in their effectiveness. There is a high sensitivity to certain animal substances, particularly certain kinds of mucus. Distinct responses have sometimes been obtained even with concentrations of 0·001 % dry weight of human saliva or the mucus washings from *Pecten*. There is evidently some specificity in the kinds of substance to which the tentacles respond. The high sensitivity to certain kinds of mucus is of great interest. This substance is often the first indication of the presence of animal food among aquatic organisms. It will, however, be noticed that different kinds of mucus differ among themselves in the extent to which they can provoke a response. Anemones themselves produce considerable quantities of mucus, to the presence of which they are quite indifferent. *Anemonia sulcata* is indifferent to its own mucus, or indeed to freshly cut-off tentacles. But if a portion of *Anemonia* is boiled for some minutes and allowed to cool, the coagulated protein mass now becomes acceptable as food, though less so than beef, or human saliva on cotton-wool. In the same way, if living pieces of *Anemonia* are fed to *Calliactis* or vice versa, feeding frequently takes place; and cases were noticed in which *Anemonia* ingested fragments previously torn off from its own body if these had decomposed.

Table 1. *Reaction to foodstuffs*

	Tentacle response. % dry wt. at limiting concentration	Relative cnida response
Human saliva	0·01	+ + +
Mucus, *Helix aspersa*	0·1	+ + +
Mucus, *Pecten maximus*	0·01–0·001	+ +
Gill and mantle, *Pecten maximus*	0·02	+ + +
Muscle, *Pecten maximus*	0·2–0·02	+ +
Liver and gonad, *Pecten maximus*	5–0·5	0
Mucus, *Loligo forbesi*	0·1–0·01	+ +
Mantle, *Loligo forbesi*	0·2	+ +
Mytilus edulis, whole animal	0·1	+ + +
Skin, *Conger vulgaris*	0·3–0·03	+ +
Muscle, *Conger vulgaris*	0·3	+ + +
Beef	0·2	+ + +
Ox blood (defibrinated)	2–0·2	0
Egg white	1·0	+ +
Gelatine	5–2	+
Egg yolk	10–5	+
Flour	10	0
Bread (not on cotton wads)	No reaction	0
'Mucus' from tube of *Myxicola infundibulum*	No reaction	0
'Mucus' from *Desmacidon fruticosum*	No reaction	0

THE CAUSE OF THE FOOD RESPONSE IN THE TENTACLES

In view of the greater sensitivity to some food substances than to others, it is not surprising that a number of localized stimuli which accompany the presence of almost any food produce no feeding reaction. Simple increase of osmotic pressure with sea water of double concentration has no effect on the tentacles; nor does a solution of high colloidal osmotic pressure such as is obtained with 10% gum arabic in sea water. Many tissues are relatively rich in potassium but even isotonic (0·6 *M*) KCl on cotton-wool wads produces no feeding reaction. The response is not brought about by hydrogen ions. Sea water at pH 4 on wads produces no effect, while even sea water at pH 1 only produces a general withering contraction of the tentacles which is quite unlike the feeding reaction. The food response to beef is equally effective whether the solution is raised to pH 9 or brought down to pH 6. Acidity does not therefore have even an indirect influence on feeding. Sea water saturated with carbon dioxide, or saturated solutions of Na_2HPO_4 also produce no effect. From this it appears that the inorganic accompaniments of food are not causing the reaction.

THE SUBSTANCES STIMULATING THE TENTACLES

When soaked into cotton wads, a suspension of beef in sea water was found to show a limit for the feeding reaction at 0·2% dry weight. The suspension was centrifuged and the supernatant fluid poured off and the flocculum resuspended in the same volume of sea water. Both supernatant fluid and resuspended flocculum produced an active feeding reaction at about half the limiting dilution of the original suspension. The high activity of the supernatant fluid shows that the active substances are not necessarily connected with the grosser solid parts of the food.

Though soluble or suspended substances from the food can produce the feeding response, these appear to be colloidal. Undiluted saliva and also beef suspension (e.g. 4% dry weight) were subjected to ultrafiltration through collodion membranes under 40 cm. of mercury. The clear ultrafiltrates produced no feeding reaction. No protein could be detected in them by the usual qualitative tests. It is noteworthy that human blood produces a strong feeding reaction (2–0·2% dry weight) but that the active substances failed to pass through the kidneys and urine is ineffective. These experiments indicate that the active food substances are either colloids or substances combined with or strongly adsorbed upon such colloids.

The obvious substances to suspect are the proteins. A beef suspension was prepared (3·5% dry weight) and made acid to pH 4·4 by the addition of acetic acid. The coagulum when washed gave a strong feeding reaction while the supernatant fluid after filtration and neutralization to pH 7·6 gave no response. The active substance had thus gone with the protein. Similarly, after prolonged boiling of a beef suspension (2% dry weight), the coagulum produced a strong feeding reaction though the clear filtrate showed none. It seems therefore that the active substance not only goes with the protein but that normally little, if any, is in free solution. It is especially remarkable that though the feeding reaction seems to involve a chemical stimulus, yet it is readily produced by apparently insoluble coagula, or even by insoluble natural products such as human skin, while washings from these produce no effect.

PROTEINS AND THEIR DERIVATIVES

When necessary, proteins and amino acids were dissolved with the aid of a little dilute sodium hydroxide and the solutions brought within the nge pH 6–9, which pH range is without influence n the response to ordinary foodstuffs. Table 2 ows the limiting concentrations for various oteins and their derivatives. Proteins have a gh activity which in many cases reaches the me order as that of natural food substances. As the latter, the activities of the proteins vary. is noteworthy that fat-free proteins appear to tivate only the tentacles and the mouth, while e cnidae are not discharged. Indeed, the disnctive feature of the pure proteins is the reonsiveness of the muscular reactions of the ntacles to these substances while the cnida sponse is almost always below normal. Subances which fail to activate the cnidae necessarily oduce much less contact between the food object nd the tentacles. Their higher limiting concenation is probably partly due to this.

At similar concentrations, peptones are slightly ore effective than the proteins from which they e derived. Amino acids are also effective Table 3). The low solubility of many amino acids akes their comparison difficult. Certain of them e especially active, notably tyrosine. The feeding action to cotton-wool soaked in amino acids is, owever, strikingly deficient in one respect. The idae are not discharged at all. This tends to ise the apparent limiting concentration. Biuret as no effect, and di- and tripeptides are not pecially potent in causing the feeding response. he experiments show that proteins themselves and their derivatives may excite the response, but that the mere presence of a peptide linkage is not enough.

Table 2. *Proteins and their derivatives*

	% dry weight at threshold concentration		Relative cnida response
	Tentacles	Mouth	
Soluble casein	0·1	0·005	+ +
Casein peptone	1·0	0·005	+
Fat-free casein	1·0	0·1	0
Egg white	1·0	0·01	+ +
Egg albumen, pure	10–1·0	1·0	0
Egg-white peptone	1·0–0·1	0·1	+
Beef	0·2		+ + +
Savory and Moore's peptone	0·1	0·005	+ +
Blood fibrin	0·4	0·005	+ +
Witte's peptone	1·0–0·1	0·01	+
Silk fibrin	No action		0
Silk peptone	Slight withering at 10%, no feeding reaction	0·005	0
Haemoglobin	1·0		0
Gelatine	5–2		+
Protein from flour	10–3		0
Gluten (wet solid)	No action		0
Sodium nucleate	5		
Sodium nucleinate	10		
Nuclein	10		

The experiments raise the question of the relation of excitation to solubility. The effects of amino acids show that substances in true solution can excite the response. But it has also been found that the active substances of natural food cannot pass through a collodion membrane and they are therefore either colloidal solutions, or in some cases actually insoluble coagula. How insoluble food substances can excite the tentacles is a matter of interest. It is possible that they exert their effect by direct contact with the sensory cells in a manner analogous to the sensitization of cnidoblasts by certain insoluble substances (Pantin, 1942).

Table 3

Amino acids, etc. dissolved in requisite alkali or acid	% dry weight at threshold concentration	
	Tentacles	Mouth
Tyrosine	1·0–0·1	0·001
Histidine	1·0	
Aspartic acid	3	0·001
l-Tryptophane	3	
Cystein	3	0·1
Glutamic acid	3	
Arginine	10	0·01–0·001
Proline	10	
Glycine	10 (tentacles wither)	1–0·01
Alanyl-glycine	10	1
Glutathione	1	0·1
Biuret (saturated solution)	No effect	

Saturated alkaline solutions of leucine, creatine, asparagine and cystine have no effect on the tentacles.

None of the substances in this table affect the cnidae.

FATS

The fact that the active agent in foods is associated with proteins and that these are themselves active does not necessarily mean that they are the only active agents. There may be others adsorbed on them, particularly lipoids. The fats obtained from dried beef, Pecten mantle or other foods by Soxhlet extraction with ether do not cause a feeding reaction. The same is true of fats extracted in the cold for 12 hr. by alcohol (2 parts) with ether (1 part). This is true both of the solid fat and of suspensions in cotton-wool made with the aid of $NaHCO_3$ solution. Pure fats such as tristearin and tripalmitin are also ineffective. Triacetin also causes no feeding response, though at concentrations of 3% or above, there is a local withering contraction of the tentacles where these have come in contact with the solution. Emulsions of fatty acids, such as

10 % oleic acid, have no effect. Lower fatty acids, like butyric, do not cause a feeding response, but bring about a strong withering contraction like that of triacetin. A 10 % solution of glycerol has no effect on the tentacles. The simpler fats and their constituents do not cause the feeding response.

Of other lipoids, cholesterol smeared on cotton-wool is without effect. Lecithin is certainly active, whether smeared on wads of cotton-wool or dispersed as an emulsion in $NaHCO_3$ solution. A true feeding response is obtained, with some clinging of the tentacles. The response of the tentacles, however, is relatively slight compared with the enormous response of the mouth to this substance. In view of the fact that two different samples of lecithin consistently showed limits of sensitivity at concentrations of about 10 and 1 % respectively, it is possible that the reaction here may be due not to lecithin itself but to impurities.

When studying the chemical activation of the cnidae (Pantin, 1942) it was found that, while fats extracted with ether were ineffective, there were other highly active lipoid substances. These remained in the food after ethereal extraction but could be obtained from the residue by Soxhlet extraction with ethyl alcohol or acetone. Pecten gill and mantle was dried and extracted with ethyl ether in a Soxhlet for 3 hr. The residue was similarly extracted with acetone, and finally with ethyl alcohol. The dried alcoholic extract easily forms a suspension in sea water with the aid of a little $NaHCO_3$. The resulting solution strongly sensitized the cnidoblasts to contact stimuli in concentrations at and above 0·3 %. The same suspension on cotton wads caused a marked feeding response, with clinging of the tentacles to the wads, at concentrations of 1·0 % or above. The extract is less active weight for weight than the gill and mantle from which it is extracted. At all concentrations it evokes a less thorough muscular response than solutions of natural foods. But if a 1 % solution of the extract is squirted locally on the tentacles, these bunch and contract as in the first stages of the feeding reaction. It seems therefore that the alcohol extract truly excites the tentacles and that the feeding reaction it produces with cotton wads is not simply due to the increased mechanical stimulus resulting from discharge of the cnidae.

CARBOHYDRATES

Maltose, saccharose, lactose, glucose, laevulose and starch produce no feeding reaction on cotton wads even in concentrations above 10 %. If the concentration of the sugar is sufficient for the osmotic pressure to be more than about twice that of natural sea water, the mouth may open if the solutions are squirted on it. This is probably due to osmotic stimulation, since high concentrations of sea water of the same order of osmotic pressure act similarly. In any case, neither the tentacles nor the cnidae show any reaction. There is, however, one carbohydrate, glycogen, which is certainly effective. Parker (1928) found that ciliary reversal of the lips of *Metridium* was caused by glycogen as well as by food. Starch and glucose, on the other hand, had no effect. In *Anemonia* a suspension of glycogen on wads was found to give a characteristic feeding reaction though with little clinging of the tentacles to the wads. The limiting concentration required to produce an effect is, however, about 10 %, so that it is far less effective than the majority of foods containing animal proteins. Further, of two separate samples of glycogen tested, one appeared to be three or four times as active as the other. It is possible that, as in the case of lecithin, the activity may be due to traces of adsorbed impurities.

OTHER ACTIVE SUBSTANCES

The derivatives of proteins and certain alcohol-soluble lipoids in food seem to be the only substances which normally evoke the feeding response in the tentacles. The fact that a substance has a high or low olfactory or gustatory activity in man gives no indication of its activity in the Actinians. We may note the high sensitivity of *Anemonia* to some kinds of mucus and its insensitivity to sugars. On the other hand, substances of high olfactory activity in man frequently have no effect. Thus $M/10$ NaSH, brought to pH 8·2, has no effect upon the tentacles, nor do the powerful substances indol and skatol. Henschel (1935) records that pieces of the manubrium of many medusae are very sensitive to the latter. But in the tentacles of *Anemonia* there is no such sensitivity. Saturated solutions, or even crystals of these substances, placed upon them have no effect at all, though squirted on the mouth they cause dilation.

There is however another response produced in the tentacles by a number of substances. It is a 'withering' retraction of the stimulated tentacle with excessive secretion of mucus. This occurs in sea water rendered acid below pH 4 with HCl, and with the lower fatty acids, acetic, butyric, valerianic, at 0·1–1 %. Triacetin at 3 % acts in the same way. Certain substances which taste bitter to man produce withering. Quinine chloride (pH 7) causes it ir concentrations of 0·3 %. A similar action of quinin is described by Nagel (1892) on the tentacles o *Calliactis* and by Henschel (1935) on the tentacle and manubrium of the hydromedusan *Sarsia* Torrey (1904) records a like effect for picric acid ir *Sagartia davisi*, and in *Anemonia*. Picric acid act in *Anemonia* at the same concentration as quinine But the most active substances for *Anemonia* appea to be bile salts and saponin. Fresh ox-gall, pur sodium taurocholate, pure sodium glycocholate, o saponin produce withering down to a concentratio of 0·1 %.

While normally this withering response does no esemble the feeding reaction, in a few cases, both vith quinine and with bile salts, a weak feeding esponse was obtained to cotton wads soaked in these ubstances just at the lowest limit of effective conentration, although controls of cotton-wool with ure sea water were rejected. But with quinine and ile salts a slight increase in concentration was ufficient to produce complete withering with no ttempt at the feeding reaction. On the other hand, vith true food substances the concentration can be aised 100- or even 1000-fold with only an increase n the speed and effectiveness of the feeding reaction. evertheless, the fact that substances in the bile salt roup can under certain conditions cause a feeding eaction indicates that the mechanisms of feeding nd withering are not altogether separate. Certain ery soluble food derivatives which can produce an nquestioned feeding response cause withering in igh concentration. This is the case with 10% olutions of glycine or of Witte's peptone. This uggests that withering is simply the result of exessive general stimulation of the tentacle. This is he more probable because it can be produced by rolonged electrical stimulation of the tentacles at frequency (e.g. 1 shock per sec.) well above that hich induces a feeding response. If the feeding eaction is due to a prolonged low frequency train f nervous impulse, and rejection to a similar shorter ain, withering may be due to a prolonged train of igher frequency.

MOUTH RESPONSE

he feeding response begins with the tentacles and completed by opening of the mouth and ingestion f food. The sensitivity of the mouth was tested by quirting about 2 ml. of a known solution on it and omparing the effect with that on the tentacles. As oeb (1895) said, the mouth is far more sensitive han any other part of the animal including the entacles. But its response is less specific and a great ariety of chemical stimuli cause it to open. Indeed, ea water concentrated to a 50% increase in osmotic ressure causes some opening, and consequently all ubstances in concentrations sufficient seriously to ncrease the osmotic pressure exert such an effect. his is the case with the sugars, saccharose, maltose, evulose, glucose and lactose, which are effective in he region of 10% concentration.

It is evident from Tables 2, 3 and 4 that the mouth not only more sensitive but that its sensitivity iffers qualitatively from that of the tentacles. There e substances like the amines and skatol to which he mouth is very sensitive which have no obvious ffect on the tentacles. On the other hand, the ensitivity to saponin and taurocholate is about qual. A similar relative variation of sensitivity is vident in food derivatives. Most of these are about n times as effective on the mouth as on the ntacles. But those amino acids which were without effect on the tentacles had no action on the mouth either. On the other hand, lecithin smeared on wads or 10% egg yolk cause great opening of the mouth but have little action on the tentacles. The mouth may normally respond to substances in the food which are not those that initiate the feeding response in the tentacles. The sensitivity is greatest however to protein derivatives, just as it is in the tentacles.

The mouth responds to food solutions by swelling and protruding as it opens just as it does to solid food. But to some substances in Table 4 the mouth opens by a contraction of the disk and mesenteries which causes the pharynx to gape in a manner not seen with food. Quinine and histamine produce this effect. This is to some extent true of the action of all the amines and physiologically related substances. For this reason and because they have no effect on the tentacles it is unlikely that they are effective agents in causing the natural food response.

Table 4

Substance	% dry weight at threshold concentration	
	Tentacles	Mouth
Quinine HCl	0·1	0·01
Na taurocholate	0·1	0·1
Saponin	0·1	0·1
Skatol	No effect	0·05
Saccharose Maltose Laevulose Glucose Lactose	No effect	10
KCl	No effect	0·5
NH_4Cl	,,	0·3
Methylamine Cl	,,	1·0
Ethylamine Cl	,,	0·1
Trimethylamine oxide HCl	,,	1·0
Histamine Cl	,,	0·2
Tyramine Cl	,,	0·2
Adrenaline Cl	,,	10·0
Choline Cl	,,	1·0
Acethylcholine Cl	,,	0·1
Tetramethylammonium Cl	,,	0·1

Most of the mouth responses are rapidly paralysed by excess magnesium, indicating that they take place through the medium of sensory structures. But quinine, histamine, strong taurocholate (10%) and sea water at *p*H 2 cause gaping opening of the mouth even in the presence of excess magnesium (50% isotonic $MgCl_2$), though the effect is then somewhat reduced. Magnesium inhibits the responses to all food substances.

CHEMICAL SENSE IN COELENTERATES

Nagel (1892) showed marked selective chemical sense in the tentacles of *Calliactis parasitica* and *Actinia equina*. Sardine meat was readily seized. while inert substances such as filter paper were rejected unless soaked in food juice. He also showed that 'sugar' failed to sensitize filter paper, while to quinine the tentacles responded by a retraction quite different from the feeding reaction. Our conclusions clearly agree with his. They are, however, contradicted by Fleure & Walton (1906). According to these authors, the feeding response of *Anemonia* tentacles is solely due to mechanical stimuli. Their evidence for this is that sometimes such stimuli alone will cause feeding; while the tentacles were only found to respond to chemical stimuli of comparatively high intensity. The latter observation is only founded, however, on responses to certain commercial food extracts, and we have shown not only that there is an unquestionable difference between the response to solid food and inert substances such as cotton-wool, but also that the latter can be activated by immersion in low concentrations of certain food solutions, notably mucus.

The power of food solutions to activate inert objects so that they are accepted as food is found not only in actinians, but also in Hydrozoa and various medusae. Beutler (1924, 1926) showed in Hydra and various Hydrozoa that food masses such as pieces of gelatine which produced no feeding reaction were rapidly ingested in the presence of juices from crushed Daphnia or the body fluids of various animals. Similarly, Henschel (1935) showed that feeding reactions could be obtained in *Aurelia aurita*, *Cyanea capillata* and *Sarsia tubulosa* to filter-paper or clay soaked in suitable food solutions.

We have shown that, in *Anemonia*, the substances which call forth the feeding response are particularly proteins and their derivatives, including amino acids. Fats are ineffective, though certain lipoids which can be extracted from food by means of alcohol, though not by ether, are also fairly active. Carbohydrates are generally without action, except in the case of glycogen, the activity of which may perhaps be due rather to adsorbed impurities than to glycogen itself. This ineffectiveness of carbohydrates was also found not only by Nagel in *Calliactis* and *Actinia*, but also by Torrey (1904) in *Sagartia davisi*.

The marked effect of proteins, as opposed to other food derivatives, is particularly characteristic of the tentacles. The mouth has both a higher chemical sensitivity and responds to a wider range of substances. Nevertheless even here, as we have shown, the reaction to proteins predominates, while there is no apparent sensitivity to sugars. In different coelenterates, a variety of different structures are involved in feeding, and as with the mouth and tentacles of *Anemonia* these do not necessarily have the same range of chemical sensitivity. However, the strong sensitivity to proteins and lack of response to carbohydrates other than glycogen appears to be fairly general. Parker (1905, 1928) found that ciliary reversal on the lips of *Metridium* took place to such substances as natural foods, Witte's peptone, and asparaginic acid. Cane sugar, glucose and other sugars, together with starch, had no effect. Glycogen, on the other hand, was found to cause reversal. Whatever the nature of this phenomenon, it is evidently controlled by a somewhat similar range of sensitivity to that controlling the responses of the tentacles in actinians.

The experiments of Henschel on the manubria of various medusae show a parallel range of sensitivity. Here again local feeding responses are obtained to proteins, peptones and various amino acids, just as they are to food solutions. Sugars and starch are remarkable for their ineffectiveness. In these animals, glycogen also is without action. On the other hand, Henschel found that the isolated mouth of *Aurelia* gave a strong positive reaction to triolein, oleic and palmitic acids, which substances are without effect on the tentacles of *Anemonia*.

Skatol, which we have seen affects the mouth though not the tentacles in *Anemonia*, has a variable effect in the medusae studied by Henschel. It has little action in *Cyanea*, more in *Aurelia*, while the manubrium of *Sarsia* is extraordinarily sensitive to its presence.

It seems therefore that though there is some individual variation, both with different species and with different parts of the animal, a number of very different coelenterates appear to be characterized by a high sensitivity to proteins and a low sensitivity to carbohydrates. On the one hand, there is an obvious correlation between this and the carnivorous mode of life of these animals. On the other, the restricted range of substances to which these animals are sensitive as compared with man should not necessarily be supposed to be due to a condition of primitive simplicity. Not only do we see cases of individual sensitivity to fats and such substances as skatol, but there seems to be some mechanism by which there is a high degree of discrimination between related chemical substances. This is notably true of mucus of prey as opposed to that of animals of the same species. There is a related phenomenon among animals commensal with anemones. Thus Thomson (1923) has shown that the crab *Stenorhyncus phalangium* normally associates with *Anemonia sulcata* and during life is untouched by the anemone. When it dies, however, its body is readily taken up and eaten.

SUMMARY

1. Feeding can be initiated in *Anemonia sulcata* by mechanical, chemical or electrical stimulation of the tentacles provided the stimulus sets up sufficiently prolonged excitation. Owing to rapid adaptation, mechanical stimuli rarely set up enduring excitation and inert objects are therefore usually rejected. Chemical stimuli set up prolonged excitation and food objects are therefore usually accepted. A series of electrical stimuli can produce rejection or feeding according to whether it is brief or prolonged.

2. The sensitivity of the tentacles varies greatly to different foods. It is greatest to animal foods. There is great sensitivity to certain kinds of mucus.

3. The active substances of natural foods are closely associated with protein. They fail to pass through a membrane which retains colloids. A feeding reaction can be obtained to food substances which appear to be insoluble in water.

4. Though the active substances of many natural foods are not in free solution, soluble derivatives of proteins, such as peptones and amino acids cause a feeding reaction. With pure proteins, the response of the cnidae is diminished. With amino acids, there is no cnida response. From this it follows that increased mechanical contact due to cnida discharge is not essential for excitation of the feeding response.

5. Fat, such as tristearin, and ethereal extracts of food are without effect. Alcoholic Soxhlet extraction of food yields a substance which causes the food reaction. Carbohydrates are without effect except in the case of glycogen, the action of which may be due to impurities.

6. The lower fatty acids, quinine and bile salts produce a withering contraction of the tentacles which differs from the response of the latter to food. The effect can be produced by prolonged electrical excitation at a higher frequency than that required for the feeding response and is therefore probably due to excessive stimulation.

7. The mouth responds to a greater variety of chemical stimuli than the tentacles. The relative sensitivity of these organs to different chemical stimuli is not the same, but for most agents the mouth is the more sensitive. Quinine, histamine and 10% bile salts excite the musculature of the mouth directly.

8. The range of chemical sensitivity in different coelenterates is discussed.

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EXPERIMENTS ON THE SILVER NITRATE METHOD FOR THE HISTOLOGICAL DEMONSTRATION OF ASCORBIC ACID (VITAMIN C)

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(With Two Text-figures)

A 10 % solution of silver nitrate in 10 % acetic acid has been widely used to show the presence of ascorbic acid in microscopical preparations (Bourne, 1936; Giroud, 1938). When there is ascorbic acid in the cells a black precipitate of metallic silver is obtained. This precipitate is often observed to be on or in the Golgi material, and it has been supposed that where this is the case the ascorbic acid is itself located in the Golgi substance (Giroud, 1938; Bourne, 1935). However, it has been pointed out that this conclusion is not necessarily correct, since the localization of the silver precipitate may be a physico-chemical phenomenon, resulting from the presence of a certain type of interface (Barnett, 1942). Experiments have therefore been carried out *in vitro* to investigate whether silver precipitates tend to accumulate at interfaces in conditions comparable with those in cells.

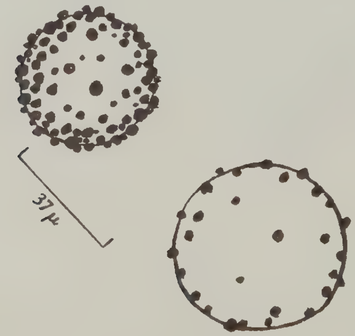

Fig. 1. Drawings of oil drops bearing silver precipitate.

To a 5 % solution of gelatin there were added a few drops of olive oil, plus crystalline ascorbic acid to give a concentration of 200 mg./100 ml. This mixture was passed through a small cream-making machine, in order to emulsify the oil, and allowed to set. Pieces of the gel were left in acid silver nitrate for about 10 min., and were then washed in distilled water; both operations were carried out in brown bottles. Thus the gel was given the same treatment as a piece of tissue in which it is intended to investigate the distribution of ascorbic acid. A small portion of the treated gel was put on a microscope slide and gently compressed under a cover-glass. Examination under the microscope showed that the silver was mainly present as granules dispersed through the gel, but some of the oil droplets had a heavy precipitate attached to their surfaces (Fig. 1): this precipitate was in the form of granules, some rather irregular in shape, and resembled that described elsewhere as occurring on the surface of the Golgi material in certain cells (Barnett & Bourne, 1942) (Fig. 2). In some instances granules also accumulated on the surfaces of what appeared to be dust particles, particularly fragments of thread; this was also observed in preparations made without olive oil.

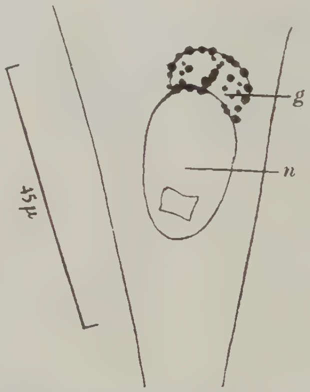

Fig. 2. Part of fibroblast-type cell with Golgi substance (*g*) bearing silver precipitate; *n* = nucleus. Redrawn from Barnett (1942).

In other preparations, instead of olive oil, ground glass or kieselguhr were added to the gelatin solution. On the kieselguhr fragments there was very little precipitate; what there was took the form of isolated granules in the interstices of the diatom skeletons.

On the ground-glass fragments there were often dense deposits.

These experiments show that even in a homogeneous medium such as gelatin solution granular precipitates can occur. The accumulation of some of the granules at the interfaces between foreign bodies and the gelatin solution must be due to processes occurring after the reduction of the silver nitrate. Consequently the form which the precipitates take in these conditions clearly has no bearing on the prior localization of ascorbic acid.

The precipitates observed in cells treated with acid silver nitrate are of three main types: first, spherical granules more or less evenly distributed in the cytoplasm; second, granules adhering to a surface, generally of the Golgi substance (Fig. 2); third, an even precipitate, not visibly granular, evidently on the surface of the Golgi material. In some instances the first type has been observed in cells which contain spherical mitochondria evenly distributed in the cytoplasm, and it has been concluded that the ascorbic acid is located in the mitochondria (Bourne, 1935). However, as we have seen, that the precipitate consists of spherical granules but this need not imply that it is attached to pre-existing structures; and in fact, in some cells a precipitate of spherical granules has been observed where the mitochondria are known to be filamentous (Barnett & Bourne, 1942). There has been no report of the observation of silver precipitates on thread-like mitochondria. It must be concluded that the evidence for the localization of intracellular ascorbic acid in mitochondria is inadequate.

With regard to Golgi material, there is little doubt that the silver precipitates obtained are often located on it: this is shown by both the shape and the position of the deposits. Those of the second type mentioned above, as the figures show, are closely paralleled by those obtained in the experiments with olive oil, but it has not been possible to imitate the third type *in vitro*. It is evidently possible that the tendency for silver precipitates to accumulate at interfaces alone accounts for the localized precipitates observed in cells. The fact that mitochondria, at least when filamentous, do not become covered with precipitated silver, may be attributed to their presenting a different type of interface to that of the Golgi substance.

It is concluded that it is unjustifiable to infer the whereabouts of ascorbic acid within the cell from the site of the silver precipitates obtained by the silver nitrate method.

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DISINTEGRATION OF EPIDIDYMAL SPERMATOZOA BY APPLICATION OF ICE TO THE SCROTAL TESTIS

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(With Four Text-figures)

The effect of temperature on spermatogenesis and on spermatozoa in mammals is very distinctive. Testicular temperature is a few degrees below body temperature, 1·5–2·5° C. lower in rabbits, 2·0–3·5° C. in guinea-pigs, 4–8° C. in rats (Moore & Quick, 1924), 4–9° C. in rams (Phillips & McKenzie, 1934). Spermatogenesis is retarded or even completely stopped by increasing the normal temperature of the testis, as for example by natural and artificial cryptorchidism (Moore, 1922, 1924 *a*, rats and guinea-pigs) or by applying heat to the testis (Fukui, 1923 *a*, rabbits; Moore, 1924 *b*, guinea-pigs; Young, 1927, guinea-pigs; Phillips & McKenzie, 1934, rams; Macleod & Hotchkiss, 1941, human). An optimum temperature, a few degrees below that of the body, can therefore be postulated for spermatogenesis. Although the contraction and relaxation of the scrotum in response to the outside temperature can bring about thermo-regulation of the testes by both raising and lowering the temperature as suggested by Crew (1922), and this mechanism undoubtedly plays a part in reducing temperature, no ill effect on spermatogenesis of lowering the temperature below the optimum has been found even when the testes of rats were subjected to a temperature as low as 6–8° C. (Phillips & McKenzie, 1934). It may be that spermatogenesis is less susceptible to injury at a lower than at a higher than normal temperature.

Spermatozoa after ejaculation survive much better at a low temperature than at a high one (Hammond, 1930; Walton, 1930). Thus a temperature of 0–10° C. is employed extensively for the storage of sperms in the practice of artificial insemination (Walton, 1933; Lambert & McKenzie, 1940). Nevertheless, spermatozoa do suffer from rapid cooling from body temperature to a low temperature (temperature shock, Milovanov, 1934) and spermatozoa survive better when cooled slowly (acclimatization) as demonstrated by Chang & Walton (1940).

The present paper reports some experimental data concerning the effect of low temperature on spermatogenesis and on epididymal spermatozoa of rabbit, rat and guinea-pig.

METHODS

The semen of rabbits was collected with an artificial vagina by the method described by Macirone & Walton (1938). The motility of the spermatozoa was examined after collection under the microscope on a warm stage. The number of spermatozoa of each sample was estimated by means of the haemocytometer technique. In order to stain the spermatozoa for counting semen was diluted with the following solution instead of with ordinary saline:

·9 % of NaCl	180 c.c.
2 % KOH	10 c.c. (for rabbit)
,,	20 c.c. (for rat and guinea-pig)
Loffler's methylene blue	3 c.c.

This solution should be freshly prepared before use. As Loffler's methylene blue contains KOH, this solution simply increases the concentration of KOH to an optimum for the staining of the spermatozoa. If the alkalinity is too strong the tails of the spermatozoa will be curled. For the sperms of the rat and guinea-pig, the alkalinity must be higher than that for the rabbit.

In this solution, spermatozoa stain blue in a few minutes. As the semen of the rabbit contains many prostatic granules, the stain is of considerable help in counting and is indispensable for counting tailless spermatozoa. The semen was diluted 1:400 in most cases. About 100 spermatozoa were counted with a hand-tally in each sample, then the number of tailless sperms in the same sample was recounted and the percentage calculated. Abnormal sperms (by which we mean those with small or abnormal heads) were counted in the same way.

Testes were treated with ice for 10 min. in the following way: A shallow groove was made in two pieces of ice. A rabbit was held in a sitting position. An assistant held the hind legs. The experimenter gently compressed the base of the scrotum so as to bring the testes into prominence and placed the two pieces of ice so as to cover the testes. In some experiments, the ice was only applied to the tail of the epididymis which can be gently pressed to the posterior part of the scrotum in the case of the rabbit. In these cases only one piece of ice was applied to the scrotum where the epididymis protruded beneath.

For rats and guinea-pigs, the animal was tied in a supine position on a bench and the ice was applied to the testis. In some experiments on rabbits, guinea-pigs and rats, only one testis was treated with ice for 10 or 20 min.; the other was pushed into the abdomen during this operation. The animals were killed 48 hr. after the treatment and the testes with the epididymis were removed immediately after death. The caput, the cauda and the vas deferens rectus were separated and macerated in a definite amount of saline, i.e. 4 c.c. for caput or cauda and 2 c.c. for vas deferens rectus. The proportion of affected and abnormal spermatozoa was counted in the usual way. The treated testis and its partner were fixed and stained simultaneously in formol thionin (Chang, 1936) and sectioned for histological examination. Although this fixation and stain was devised for nerve tissue it was employed in this study because of simplicity. It is quite satisfactory for a general examination of spermatogenesis.

In another series of experiments, a freshly collected sample of rabbit semen from which first of all 0·1 c.c. was taken for counting, was quickly cooled in ice for 20 min. then held in a chamber at 32° C. for 24 hr. The number of spermatozoa and the number of tailless sperms were again counted.

The respiration of spermatozoa collected from the epididymis of rabbit, and rat 48 hr. after treatment with ice, was measured in a Barcroft differential respirometer (Dixon, 1934). In measuring respiration, sperms were diluted in a solution containing Na_2SO_4 0·36 g., glucose 3·9 g., water 100 c.c. (Walton, 1933).

RESULTS

The results are graphically represented in Figs. 1–4. It is very clear that the proportion of tailless sperms increased very markedly within 24 hr. of the application of ice through the scrotum to either testes and epididymis or to epididymis alone. Ten days after the treatment, the normal proportion of tailless sperms in the semen reappeared. The motility of the sperms was affected in correspondence with the proportion of tailless sperms; when there was a high percentage of tailless sperms there were only a few motile sperms in the sample. Ten days after the treatment, the proportion of motile sperms increased as the proportion of tailless sperms decreased. There was no adverse effect of the treatment on the sex drive even when treated several times and the animals reacted normally to the 'dummy'. Four rabbits reacted to the 'dummy' and ejaculated very well even 1 hr. after treatment. Another rabbit (no. 50) was treated seven times within three months, and he reacted and ejaculated very well without any difficulty. There was no effect on the volume of the semen or on the gelatinous secretion which often occurs in the semen of the rabbit. The proportion of abnormal sperms in the semen was also not influenced by the treatment as the curves show.

From previous experience, we have found that the total number of sperms ejaculated is subject to great variation. After a long sexual repose it is always higher than that collected afterwards (see Figs. 1–3). The total number of sperms ejaculated depends also on the number of collections (see Fig. 3). Taking these variables into account the data accumulated in this experiment disclose no sign of any adverse effect of ice treatment on sperm production or spermatogenesis. Rabbit 50 was treated seven times in three months (Fig. 3 represents only the data of the last treatment), and the number of spermatozoa did not decrease. Furthermore, as there was no increase of abnormal sperms, spermatogenesis appears to be unaffected. Histological examination of the testes revealed that normal spermatogenesis occurred in the treated testis and its untreated partner.

The following facts indicate that disintegration of spermatozoa on application of ice takes place in the epididymis: (1) Tailless sperms appeared in large numbers within 24 hr. of treatment. If the effect was on the spermatozoa in the testis or rete alone a much longer interval would elapse before their appearance in the ejaculate. (2) The sperm heads and the sperm tails examined under the microscope are morphologically mature and full maturity is reached only in the epididymis. (3) The high proportion of tailless sperms recovered from the macerated epididymis shortly after the ice treatment, as shown in Table 6, clearly reveals that disintegration takes place in the cauda epididymis.

The disintegrated sperms appeared in the ejaculate about 8–24 hr. after the treatment and lasted for about 10 days when the collections were made frequently (Table 1 and Fig. 4). If the collections were made less frequently, their occurrence lasted longer (Table 2). This clearly indicates that the effect occurs in the cauda epididymis where the sperms are stored and that the rate at which recovery takes place depends on the rate of evacuation.

The complete morphological disintegration of the epididymal sperms after application of ice to the testes through the scrotum requires a few hours to come to completion as shown in Table 1 and Fig. 4. There is no increase of tailless sperms from ejaculates 1–2 hr. after treatment. This is not due to reserve of unaffected sperms in the vas deferens rectus, because the number of sperms stored in the rectus is less than the number of sperms ejaculated before tailless sperms appeared as shown in Table 3. The motility of sperms from the first ejaculate 1–2 hr. after the treatment was very good (the ejaculate contains the sperms from the vas deferens), but the motility of sperms from the second or third ejaculate 1–2 hr. after treatment was very poor. This indicates that physiological deterioration has preceded morphological disintegration and that physiological deterioration sets in very rapidly. This is also borne out by the evidence from a rabbit (no. 90 in Table 6) which was killed 1 hr. after treatment. Physiological deterioration in the treated testis is shown by the marked reduction in motility, while morphological disintegration is not conspicuous.

Table 1. *The occurrence of tailless sperms in relation to time (percentage of tailless sperms and percentage of abnormal sperms in ejaculates after application of ice to the testes)*

Rabbit no.	Before treatment		1 hr. after		2 hr. after		8 hr. after		1 day after		2 days after	
	Tail-less	Ab-normal	Tail-less	Ab-normal	Tail-less	Ab-normal	Tail-less	Ab-normal	Tail-less	Ab-normal	Tail-less	Ab-normal
16	5	0	8	6	—	—	25	12	27	7	—	—
50	6	12	12	2	10	10	—	—	90	0	95	6
53	1	1	2	4	—	—	22	12	24	4	—	—
90*	7	9	—	—	9	4	—	—	34	9	—	—
92	5	5	12	2	—	—	—	—	64	5	—	—
Mean	5	5	9	4	10	7	24	12	48	5	95	6

Rabbit no.	3 days after		4 days after		6 days after		8 days after		10 days after	
	Tail-less	Ab-normal	Tail-less	Ab-normal	Tail-less	Ab-normal	Tail-less	Ab-normal	Tail-less	Ab-normal
16	—	—	20	4	—	—	—	—	—	—
50	—	—	70	5	41	2	24	5	—	—
53	—	—	57	10	—	—	—	—	—	—
90*	38	12	—	—	22	8	18	10	8	3
92	41	6	—	—	—	—	34	8	12	3
Mean	40	9	49	6	32	5	25	8	10	3

* Application of ice to the epididymis.

Table 2. *The time of recovery after treatment and its dependence upon the frequency of collections of semen*

Rabbit no.	No. of collections	Time of recovery in days
50	4	16
	4	22
	19	12
90	9	14
	11	11
	11	10
92	7	15
	9	10
	11	10

Table 3. *Total number of sperms in vas deferens rectus and total number of sperms in the ejaculates before the occurrence of disintegrated sperms*

	Total no. of sperms recovered in the two vasa deferentia in millions	Total no. of sperms in the ejaculates after treatment but before the occurrence of tailless sperms in millions
Average	46	103
Range	17–78	36–246
No. of rabbits	3	5

The proportion of disintegrated epididymal spermatozoa depends on the duration of treatment, i.e. the longer the application of ice to the scrotal testis the higher the percentage of disintegrated spermatozoa as shown in Table 4.

It is interesting that the sperms in the ejaculated semen suffer from low temperature treatment far less than the sperms in the epididymis as shown in Table 5. 15–95 % of tailless sperms were found after treatment of the testes with ice while only 4–9 % were found after treatment of the semen with ice (Tables 1, 5 and 6). It seems that the accessory secretions may have some capacity to protect spermatozoa against the disintegrating effect of low temperature. However, rapid cooling does injure the physiological integrity of sperms in the semen of the ram as demonstrated by Chang & Walton (1940).

Tables 6 and 7 represent comparative results on rabbits, rats and guinea-pigs after treatment with ice on one testis and compared with the untreated testis. The percentage of tailless sperms recovered from the cauda epididymis of rabbits ranges from 15 to 59, whereas that of tailless sperms recovered from ejaculates ranges from 24 to 95 (Tables 1 and 6). The percentage of tailless sperms recovered in the

Table 4. *Disintegration of epididymal spermatozoa in relation to the duration of ice treatment of testis*

Animals	Duration of treatment in min.	Percentage of tailless sperms	Remarks
Rabbit 16	10	27	Sperms in ejaculate 24 hr. after treatment
	20	81	
Rabbit 92	10	64	
	20	86	
Rat 1	10	10	Sperms in cauda epididymis 48 hr. after treatment
Rat 3	15	33	
Rat 2	10	11	
Rat 4	15	26	

Table 5. *Percentage of tailless sperms and percentage of abnormal sperms after ice treatment of ejaculates*

Rabbit no.	Fresh semen		The same samples treated with ice	
	% tailless	% abnormal	% tailless	% abnormal
51	2	8	4	5
52	3	1	9	3
53	2	1	8	6

Table 6. *Sperm content in sections of the vas deferens after application of ice on one testicle through scrotum*

(48 hr. after treatment except rabbit 90 which was killed 1 hr. after treatment)

Animals	Treatment	Caput epididymis			Cauda epididymis			Vas deferens rectus		
		Total no. in millions	% tail-less	% ab-normal	Total no. in millions	% tail-less	% ab-normal	Total no. in millions	% tail-less	% ab-normal
Rabbit 49	Treated 10 min.	7	3	10	188	59	5	6	62	4
	Untreated	17	3	16	104	4	6	11	4	8
Rabbit 52	Treated 10 min.	48	2	18	300	15	4	30	8	6
	Untreated	24	0	16	272	1	7	48	2	6
Rabbit 90	Treated 10 min.	20	10	20	244	15	10	22	7	14
	Untreated	32	7	8	264	8	6	10	10	10
Rat 1	Treated 10 min.	33	21	—	72	10	—	14	20	—
	Untreated	48	15	—	86	1	—	8	5	—
Rat 2	Treated 10 min.	28	12	—	96	11	—	8	28	—
	Untreated	24	13	—	160	2	—	10	2	—
Rat 3	Treated 15 min.	18	9	—	58	33	—	3	18	—
	Untreated	28	9	—	46	0	—	1	0	—
Rat 4	Treated 15 min.	21	8	—	74	26	—	9	19	—
	Untreated	34	5	—	126	1	—	7	5	—
Guinea-pig 1	Treated 15 min.	0·8	20	—	25	4	—	0·2	0	—
	Untreated	0·3	0	—	13	0	—	1·5	3	—
Guinea-pig 2	Treated 20 min.	1·2	40	—	33	9	—	0	0	—
	Untreated	0	0	—	13	3	—	3·5	6	—
Guinea-pig 3	Treated 20 min.	1·8	60	—	166	4	—	7·8	1	—
	Untreated	1	30	—	143	2	—	13·2	2	—

Table 7. *Summary of percentage of tailless sperms in the sections of the vas deferens of different species*

Animals	Treatment	Caput epididymis	Cauda epididymis	Vas deferens rectus
Rabbits	Treated	5	30	26
	Untreated	3·3	4·3	5
Rats	Treated	12·5	20	21·3
	Untreated	10·5	1	3
Guinea-pigs	Treated	40	5·7	0·3
	Untreated	10	1·7	3·7

cauda epididymis of rats ranges from 10 to 33 and that in the cauda epididymis of guinea-pigs ranges from 4 to 9. Comparing the proportion of tailless sperms from treated and untreated epididymis, there is a significant difference in the case of rabbits and rats but not in the case of guinea-pigs. However, the percentage of tailless sperms recovered in the caput epididymis of guinea-pigs is rather high as compared with untreated specimens. It may be that, in the case of the guinea-pig, the effect of low temperature is on the spermatozoa in the caput instead of in the cauda epididymis. The high proportion of tailless sperms recovered in the vas deferens rectus after treatment of the testis in the case of the rabbit and rat (48 hr. after treatment) may indicate a rapid transportation throughout the tract in sexual repose and that the actual site of damage may be higher up the tract than the observation would appear to indicate.

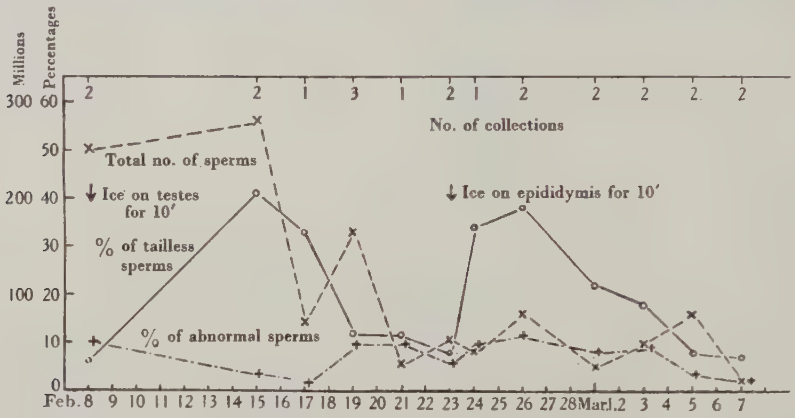

Fig. 1. Sperms of Buck 90 before and after treatment.

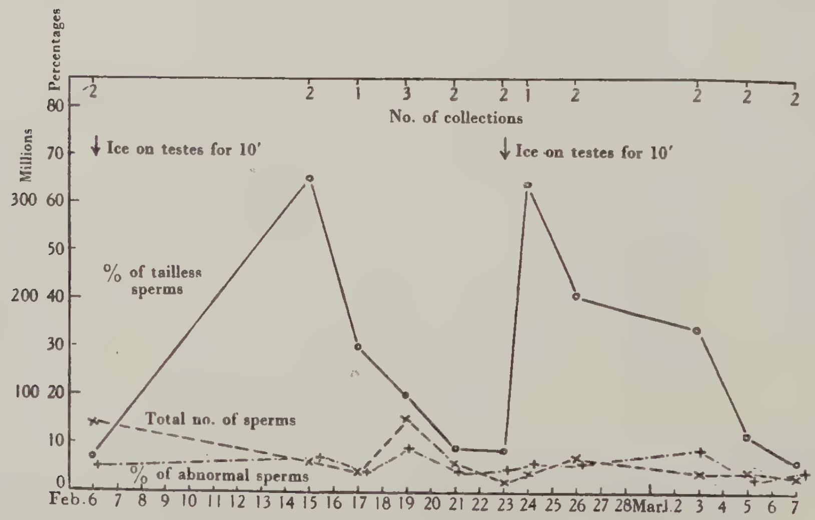

Fig. 2. Sperms of Buck 92 before and after treatment.

The epididymal sperms of different species may have different resistance to low temperature, but the morphological constitution of the scrotum may play an important part. The scrotum of the guinea-pig is thicker and not so pendulous as that of the rat and the rabbit, and the cauda epididymis is very closely attached to the testis so that the temperature actually received during treatment may not be low enough to produce such an effect as in the case of rabbit and rat, the caudae of which are less closely attached to the testis. These may be the reasons why negative results were obtained in the guinea-pigs even though the treatment was longer, i.e. 20 min. The scrotum of the rabbit is very thin and the cauda epididymis is not very closely attached to the testis and the experimental results are more striking.

The results may also be influenced by the number of spermatozoa actually present in the cauda at the time of treatment. If the cauda is very full of spermatozoa the wall of the duct will be distended and thinner and offer less protection against cold. The scrotum of rabbit 50 was very thin and this rabbit produced more sperms than the others and gave the highest percentage of tailless sperms after treatment.

From a few determinations made, the respiration of spermatozoa taken from the epididymis of the rabbit and rat, 48 hr. after ice treatment of the testis, is very low in comparison with those taken from the untreated epididymis.

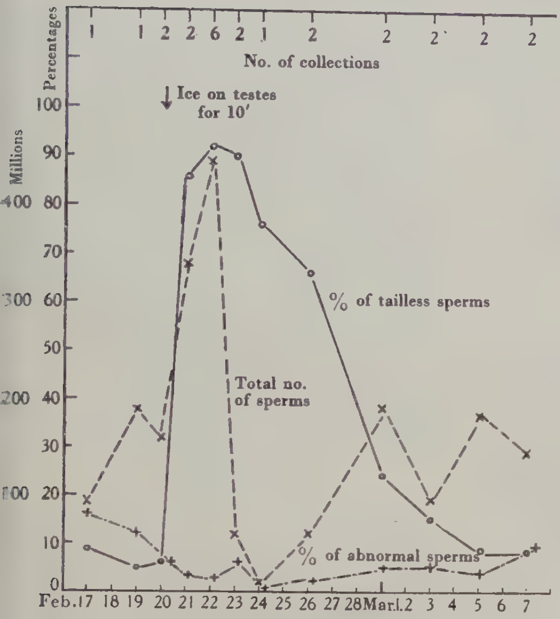

Fig. 3. Sperms of Buck 50 before and after treatment.

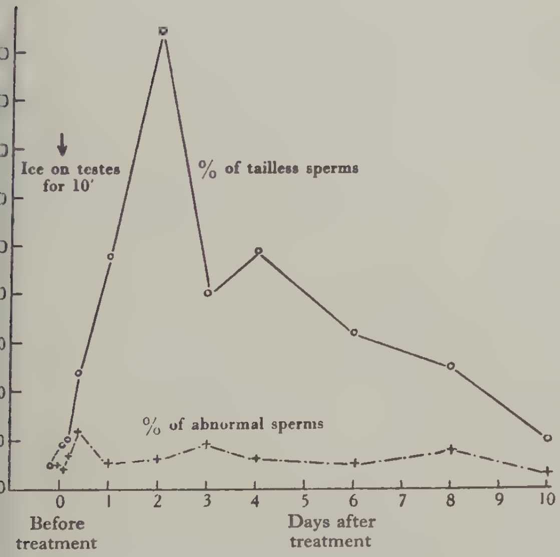

Fig. 4. The occurrence of tailless sperms and abnormal sperms in relation to time, mean of all experiments.

DISCUSSION

The method used to apply cold to the testes in these experiments does not allow very accurate control. There are differences in the thickness of the scrotum, differences in the extent to which the epididymis is adherent to the testis in different animals and differences in the extent to which the cauda is distended with spermatozoa in different individuals. The vascular circulation during the application of ice may also have varied and could not be controlled. Should the arteries to the testis have been compressed during the operation the temperature of the testis and epididymis may have been lower than when the arteries were not compressed. There can be no doubt however that the effect of low temperature on the epididymal spermatozoa is a real one.

The effect of a higher temperature on spermatogenesis is well established. On the other hand, spermatogenesis is not known to be affected by low temperature and this has not been shown in the present experiment. It is rather a striking phenomenon that spermatogenesis is retarded or stopped at body temperature, only a few degrees higher than the scrotal temperature, but not adversely affected even at a temperature far lower than body temperature. Fukui (1923*b*) believes that the heat degeneration is due to lability of testicular proteins at body temperature or above, while Moore (1924*b*) suggests that such degeneration may be due to a lack of oxygen and accumulation of carbon dioxide due to vascular stagnation. Heat applied to the scrotum, or directly to the testis produces, as in other parts of the body, a hyperaemic condition associated usually with oedema. Barron (1933), studying the effect of section of the external spermatic nerve, suggests that the degeneration of spermatogenesis that follows is due to hyperaemia resulting from vaso-dilation, rather than to the small increase in temperature. Considering these facts, Phillips & McKenzie (1934) are in favour of the view that testicular degeneration at high temperature may be due to hyperaemia rather than to the lability of testicular proteins at high temperatures.

Since a large number of different chemical and physical reactions are concerned with vital processes and since these reactions may have very different temperature coefficients, lowering the temperature might bring about a disturbance of the normal equilibrium within the cell, but why this should affect the mature spermatozoa and not spermatogenesis is unknown. A more fundamental investigation of the biochemistry of spermatogenesis and the spermatozoa might reveal the underlying processes.

Mature spermatozoa before ejaculation are stored in the cauda epididymis. The temperature here is lower than that of other parts of the testis as reported by Krainer (1934). This may afford a more suitable temperature for the survival of mature spermatozoa before ejaculation. Although the optimum temperature may be below that of the body, the epididymal spermatozoa are nevertheless sensitive to a sudden fall of temperature as the experiment shows. This effect may be the same or similar to the temperature shock demonstrated by Chang & Walton on ejaculated sperms of rams (1940).

In rabbits, the proportion of tailless sperms in the ejaculates after treatment is higher (95–24 %) than that of tailless sperms taken from the epididymis (59–15 %). Disintegration of the spermatozoa takes a few hours as the results show. We know from Young (1931) that regressive or degenerative changes of spermatozoa follow the attainment of an optimal functional capacity in the epididymis, and that these changes take place in the vas deferens or in the ampulla of Henle. It is very reasonable to suppose that the disintegration of sperms whether normal or after treatment of ice on the testis is more extensive during their passage through the vas deferens.

All epididymal spermatozoa are not equally disintegrated by the application of ice, e.g. about 50 % are affected in the case of the rabbit. It is possible that age of formation is responsible for this variation and that 'old' spermatozoa are more susceptible to disintegration.

The transportation of spermatozoa from cauda to vas deferens rectus is a continuous process even if the animal is at sexual repose; correspondingly there is a high proportion of disintegrated sperms in the vas deferens rectus when after sexual rest the animal was killed 48 hr. after treatment.

Whether treatment of the testes with ice as in this experiment would temporarily render the animal infertile can be tested in further experimentation, but it is safe to predict that fertility will be impaired.

SUMMARY

Testes of rabbits were cooled with ice applied to the scrotum for 10 min. Disintegrated spermatozoa (95–24 %) appeared in the ejaculates 24 hr. after treatment. Morphological disintegration takes a few hours but physiological deterioration appears rather more quickly. The proportion of disintegrated spermatozoa depends on the duration of treatment. The occurrence of disintegrated sperms in the ejaculate lasted about 10 days, depending on the rate of evacuation of disintegrated sperms from the cauda epididymis. There was no adverse effect of ice treatment on sex drive and spermatogenesis. The testes of rabbits, rats and guinea-pigs were treated with ice for 10–20 min. About 20–30 % of disintegrated sperms was found in the cauda epididymis of rabbits and rats but not in guinea-pigs.

The writer wishes to acknowledge his thanks to Dr J. Hammond for giving him the opportunity to carry out this experiment; to Dr A. Walton for his constant encouragement and some valuable suggestions and to the Agriculture Research Council for a grant.

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ll be stated, refers to a group of tests performed ultaneously.

t is easy to assess the result of a test if the diet is irely successful or entirely unsuccessful. In the rner case the mortality is low and the rate of wth similar to that on wholemeal flour. In the er case little or no growth takes place and the vae die while still small. If a diet is only partly icient the rate of growth is retarded and the rtality increased. The most significant figure for ssing rate of growth is the duration of larval life il pupation. The methods of assessing results are y described elsewhere (Fraenkel & Blewett, 3*c*). For purposes of summarizing results the owing symbols are used:

+ + + +	optimum growth resembling wholemeal flour cultures.
+ + +	slightly less effective than on wholemeal flour.
+ +	still less effective than on wholemeal flour.
+	mortality generally high and development slow.
±	very unfavourable; development may be completed after long delay by one or two specimens.
–	no specimen completes development.

Most of the results are graphically summarized in ves in which the total number of pupae or adults med is plotted against time. With an optimal diet wth is fast, the variations between individuals are tively small, and the mortality is low. The curves refore are steep and reach a high level. As diets ome more unfavourable, growth becomes reded, the variations between individuals become ger and the mortality rises. The growth curves refore become shifted to the right, they rise more dually and only reach a low level. Unfortunately, curves cannot express the result of totally unourable diets when the larvae die before reaching pupal or adult stage.

Table 1

Whole diet: casein 50 parts, starch or glucose 50 parts, cholesterol 1 part, yeast 5 parts, water 15 parts.

	Tribolium	*Silvanus*	*Lasioderma*	*Sitodrepa*	*Ptinus*	*Ephestia*
Vholemeal	+ + + +	+ + + +	+ + + +	+ + + +	+ +	+ + + +
tarch diet	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ +
tarch diet without cholesterol	+ + +	+ + +	+ + +	±	+ +	–
Glucose diet	+ + +	+ +	+ + +	+ + + +	+ + +	±
Glucose diet without cholesterol	+ +	+ +	+ + (+)	+ (+)		–
Vithout carbohydrates	+ (+)	–	+ +	±	+ +	–
Vithout yeast	–	–	–	–	–	–

THE BASIC FOOD REQUIREMENTS

(Figs. 1–5 and Table 1)

e composition of the synthetic diet is described p. 28. When the carbohydrate used is starch this diet is just as good as wholemeal flour for *Tribolium*, *Silvanus*, *Sitodrepa* and *Lasioderma*. *Ptinus* grows on the starch diet very much better than on wholemeal flour. This is probably due to its higher content of B vitamins, supplied in 5% yeast. For *Ephestia* the synthetic diet was greatly inferior to wholemeal flour.

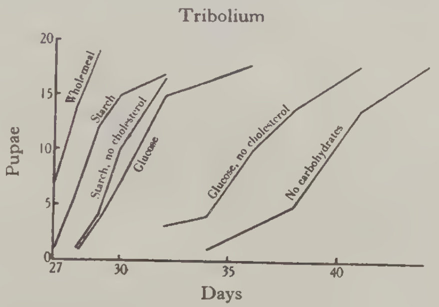


Fig. 1. *Tribolium confusum*. Growth on a 'synthetic' diet consisting of casein, glucose or starch, yeast, cholesterol, salts and water, and on the same diet minus carbohydrate or cholesterol. No growth takes place on the diets without yeast.

With glucose substituted for starch the efficiency of the diet remains unaltered for *Sitodrepa*, while growth of *Tribolium*, *Lasioderma*, *Silvanus* and *Ptinus* is somewhat retarded. But on other occasions, *Tribolium*, *Lasioderma* and *Silvanus* grew on a glucose diet just as well as on wholemeal flour. *Ptinus*, on the other hand, always seemed to grow better on starch than glucose. *Ephestia* grew badly on glucose diets and presented unaccountable difficulties.

The next step in the analysis was to find out whether all the ingredients of the 'complete diet' were essential for the insects tested. Accordingly, carbohydrate, yeast and cholesterol were left out from the diet in separate tests. In the case of yeast and cholesterol which only constitute a small proportion of the diet, the proportions of the remaining substances remained unchanged, which only very slightly alters its percentage composition. In diets without carbohydrates the amount of casein was made up to 100 parts.

When carbohydrates were omitted from the diet, the rate of growth was generally depressed, which one might have expected of insects living normally on a medium so rich in starch (*c.* 75 %) as flour. There were, however, spectacular quantitative differences. *Tribolium, Lasioderma* and *Ptinus* developed noticeably more slowly but still satisfactorily, and the mortality was not increased. *Sitodrepa* grew very slowly, with only three larvae out of twenty completing development. *Silvanus* and *Ephestia* entirely failed to grow without carbohydrates and died as small larvae.

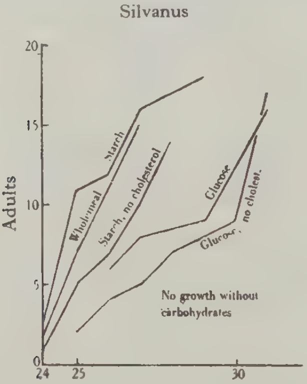


Fig. 2. *Silvanus surinamensis.* Explanation as Fig. 1.

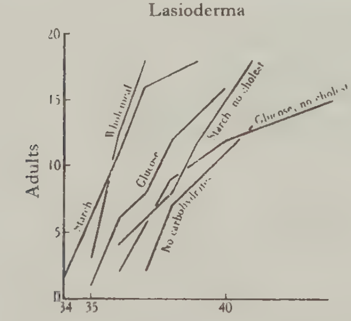


Fig. 3. *Lasioderma serricorne.* Explanation as Fig. 1.

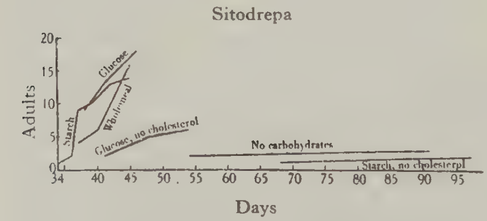


Fig. 4. *Sitodrepa panicea.* Explanation as Fig. 1.

Without cholesterol, both starch and glucose diets became adversely affected but to a very different degree for the different insects. With *Silvanus* the difference was hardly noticeable; it was slight but definite with *Tribolium* and *Lasioderma*. With *Ptinus* the gap between the efficiency of cholesterol-free and cholesterol-containing diets became still wider.

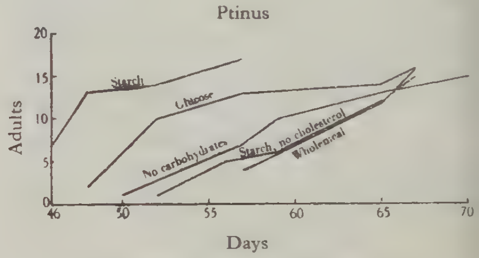


Fig. 5. *Ptinus tectus.* Explanation as Fig. 1.

Sitodrepa grew very slowly without cholesterol and few larvae completed development, while *Ephesti* entirely failed to grow. From this result it appear that the difference in sterol requirements is less o a qualitative than a quantitative nature, and this wil be further analysed below. The cholesterol-free diet contained some ergosterol as a constituent of th yeast in quantity sufficient or almost sufficient fo some of the insects and insufficient for others.

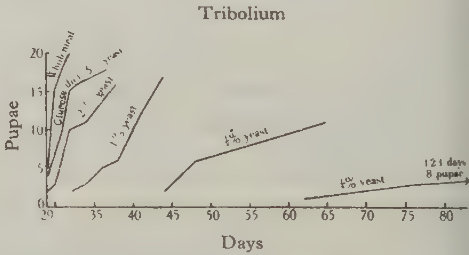
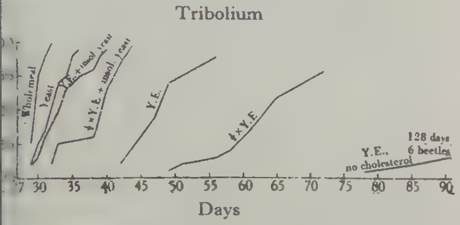


Fig. 6. Growth of *Tribolium confusum* on a diet con sisting of casein, glucose, cholesterol, salts and wate with the addition of graded quantities of dried brewer yeast.

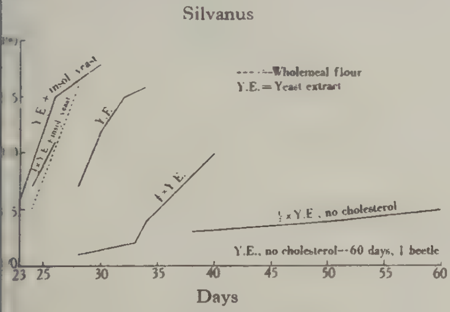
Without yeast none of the insects grew and deat occurred at an early stage. Fig. 6 shows that th amount of yeast required for optimal growth *Tribolium* is probably not very much less than th 5 % included in the diets. With 2½ % the diet b comes slightly and with smaller amounts increasing inferior.

Subsequently the constituent yeast was split into water-soluble and water-insoluble fractions. The er-soluble extract was prepared according to ck & Roscoe (1930) from fresh brewers' yeast ching yeast, supplied by Nicholson's Brewery, idenhead) and was concentrated so that 1 c.c. responded roughly to 1 g. of dry yeast. It was d undiluted. 'Insoluble yeast' is prepared by ing fresh brewers' yeast in a large volume of er, filtering and repeating the process nine times. diet then had the following composition:

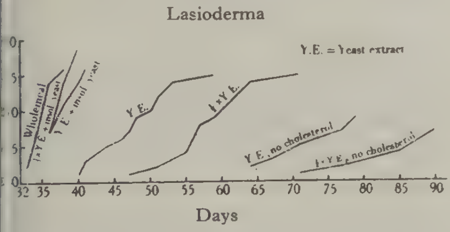
ein	50	Cholesterol	1
cose	50	Yeast extract	15
Collum's salt mixture	1	Insoluble yeast	5



. 7. *Tribolium confusum.* Growth on a diet consisting casein, glucose, cholesterol, salts and water, with e addition of either yeast, or yeast extract + insoluble sidue of yeast, or yeast extract alone, or yeast extract one without cholesterol. *Y.e.* = yeast extract. '½ yeast tract' indicates half the quantity of yeast extract.



g. 8. *Silvanus surinamensis.* Explanation as Fig. 7.



g. 9. *Lasioderma serricorne.* Explanation as Fig. 7.

his diet was compared with the original diet which contained yeast instead of the separated soluble and insoluble fractions of yeast, and with diets from which either insoluble yeast alone or in addition cholesterol, was omitted. The results are given in Figs. 7–11 and are summarized in Table 2. It is seen that splitting yeast up into its soluble and insoluble components does not influence the efficiency of the diets. Without insoluble yeast the diet becomes appreciably less efficient. It is shown elsewhere (Fraenkel & Blewett, 1943*b*) that this diet is deficient in an important growth factor, biotin. Leaving out both insoluble yeast and cholesterol, the diet becomes entirely, or almost entirely, inefficient, which demonstrates conclusively the importance of a sterol for all the insects tested. Yeast extract cannot be considered entirely free of ergosterol, and it seems probable that these traces were enough to allow some slow growth of *Tribolium*, *Lasioderma* and *Silvanus*. No growth takes place on diets which contain insoluble yeast but no yeast extract.

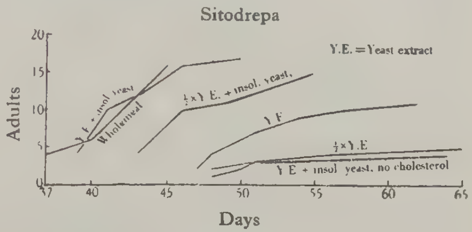


Fig. 10. *Sitodrepa panicea.* Explanation as Fig. 7.

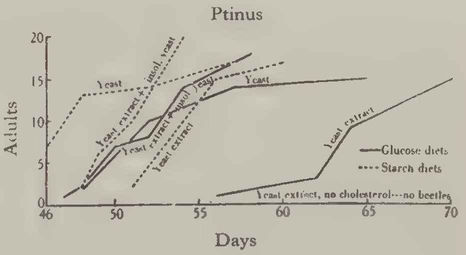


Fig. 11. *Ptinus tectus.* Broken lines refer to diets containing starch in place of glucose. Explanation as Fig. 7.

A diet containing the water-soluble and insoluble fractions of yeast was at first not as efficient as a diet containing whole yeast. The amount of yeast extract used was calculated to correspond to the quantity of yeast from which the extract had been prepared. Since in the original glucose-casein-yeast diet yeast constituted about 5%, i.e. 100 mg. in 2 g. of food, the corresponding quantity of yeast extract would be 0·1 c.c., in which 1 c.c. corresponds to 1 g. of dry yeast. Actually 0·15 c.c. was used at first, but on this, with insoluble yeast added, *Tribolium* grew noticeably slower than on whole yeast. Doubling this quantity of yeast extract restored the efficiency of the yeast extract + insoluble yeast approximately to that of whole yeast. This is shown in Figs. 7–19 where '½ yeast extract' indicates half the quantity of yeast extract which was ultimately used. This is easily explained. Preparation of the yeast extract implies only 15 min. boiling of the washed yeast in an open pan. It cannot be expected that this treatment would bring the water-soluble vitamins quantitatively into solution, neither is it likely that the proportion of the individual vitamins in solution would then be the same as in the original yeast.

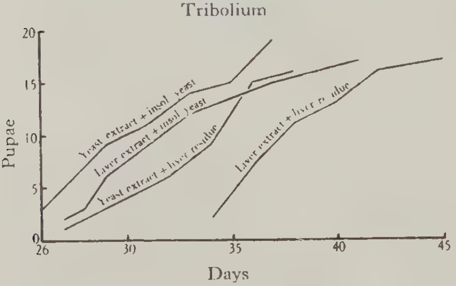


Fig. 12. Growth of *Tribolium confusum* on diets containing various combinations of the water-soluble and the water-insoluble fractions of yeast and liver.

Table 2. *Effect of modifying certain components of the synthetic diet*

Basal diet: casein 50 parts, glucose 50 parts, salts 1 part, cholesterol 1 part (except last line).

Additions to basal diet	*Tribolium*	*Lasioderma*	*Silvanus*	*Sitodrepa*	*Ptinus*
Yeast	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Yeast extract + insoluble yeast	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Yeast extract	+ +	+ +	+ + (+)	+ +	+ +
Yeast extract, no cholesterol	+	+	+	–	–

Yeast extract can be successfully substituted by other rich sources of B vitamins such as liver extract* (Fig. 12) or dried egg, and insoluble yeast can be substituted by the insoluble residue of liver. The combinations, liver extract + insoluble liver, liver extract + insoluble yeast, and yeast extract + insoluble liver are of similar efficiency to yeast extract + insoluble yeast (Fig. 12). An accurate comparison of these various liver-yeast combinations is not possible because the yeast and liver preparations were of different strength and it is not obvious on which basis a quantitative comparison should be made. It is, however, clear from the curves of Fig. 12 that liver extract is roughly qualitatively equivalent to yeast extract, and insoluble liver to insoluble yeast.

DISCUSSION

From the results reported in this paper it is clear that all the insects under investigation require cholesterol, and substances contained in the water-soluble and water-insoluble fractions of yeast. They differ, however, in their requirements of carbohydrates. The sterol requirements of the insects in question are further and more fully described and discussed elsewhere (Fraenkel & Blewett, 1943*a*). Many authors have shown before the need of insects for water-soluble vitamins of the B group (which would be contained in a yeast extract), but the need of insects for a substance contained in the insoluble part of yeast has only very recently been recognized (Fröbrich, 1939; Tatum, 1941). A full analysis of the growth factors in yeast, which are required by the insects in question, is given elsewhere (Fraenkel & Blewett, 1943*b*, *d*), where it is shown that *Tribolium* and *Ptinus* require certainly B_1, riboflavin, nicotinic acid, B_6, pantothenic acid, choline, and possibly inositol and *p*-amino-benzoic acid. In addition t these water-soluble factors biotin has been determined as the factor required from insoluble yeast.

The differences with which our insects react to th absence of carbohydrates from the diet requires som comment. While it was to be expected that insect for which flour is a natural food would grow well o a diet which contains large quantities of starch, i has never been investigated whether carbohydrate constitute an essential constituent of the diet of suc insects. From the data given in this paper it is clea that some of the insects tested require carbohydrate and others not. From this we would expect the latte group to be able to grow on a much wider variety c natural foods than the former. All the six insects i question are common pests on stored food, and th literature abounds in statements on the occurrenc articular insects on particular foods. While many hese statements must be treated with reserve it vident that the presence or absence of carbo- rates in the food appears to be one of the decisive ors for the specificity of a food for different ects. *Ephestia kuehniella*, *Sitodrepa* and *Silvanus*, ch do not grow without carbohydrates, seem ex- ively to infest food with a high carbohydrate con- The occurrence of *Ephestia* is limited to grain ereal products like flour, bran, rolled oats and aroni. *Sitodrepa*, in addition to these foods, is a on dry bread, rusks and biscuits, and *Silvanus* is wn to infest dried fruit which has a very high r content. The distribution of the three species ch grow in the absence of carbohydrates is very h wider. While occurring in much the same al products they also occur on food with a very carbohydrate content like yeast (*Tribolium*, *us*), tobacco (*Lasioderma*), fish-meal, dead insects, e casein, meat-meal (*Tribolium*, *Lasioderma*, *us*). A full analysis of the nutritional relations ween stored-product insects and their natural d is given elsewhere (Fraenkel & Blewett, 1943*c*).

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* Kindly supplied by Glaxo Laboratories.

he fact that *Tribolium* does not require carbo- rates in the diet has been curiously overlooked other authors. Chiu & McCay (1939), in an mpt to investigate the food value of cane sugar *Tribolium*, came to wrong conclusions through ing to recognize that growth is possible without ohydrate. *Tribolium* has recently been used by eral authors in nutritional experiments, and it is ful to compare the composition of the diets used ble 3).

Table 3. *Synthetic diets for* Tribolium confusum

	Fröbrich	Offhaus	Chiu & McCay	Barton-Wright	Rosenthal & Reichstein	Fraenkel & Blewett
rotein	15 % casein	20 % fresh egg white	20 % casein	15 % casein	20 % casein	45 % casein
arbo- ydrate	70 % rice starch	76 % rice starch	67 % maize starch	75 % starch	75 % rice starch	45 % glucose
at	5 % cotton-seed oil	1 % ground-nut oil	5 % cotton-seed oil	3 % fat		None or ½ % wheat-germ oil
holesterol	—	—	—	—	—	1 %
east	5 %	1–5 %	5–10 %	1–10 %	—	5 %
alts	5 % Osborn-Mendel	$3\frac{1}{2}$ % K_2HPO_4	3 % Osborn-Mendel	4 % Osborn-Mendel	5 % McCollum	1 % McCollum

he composition of the diets, given by different hors in different units, is expressed in this table comparable percentages. The figures are only ghly true. These diets differ mainly in the fol- ing points:

1) The other authors had starch for the main redient of the diet, using it in proportions similar hose in flour. It is well known that starch cannot btained in the pure state and that certain vitamin ciencies only become manifest when sugar is given instead of starch. Glucose was used by us as the carbohydrate in all the vitamin-deficiency tests to be described in the following papers (Fraenkel & Blewett, 1943*b–d*).

(2) We did not know whether 15–20 % casein would be sufficient for all the insects which we were using or whether casein would optimally satisfy the protein requirements of our insects. To be on the safe side the quantity of casein was increased, and of carbohydrate correspondingly reduced to about 45 % each of the dry diet.

(3) The difficulty of measuring out the salts into the diets with sufficient accuracy (p. 28) made it desirable to reduce the quantity of salts in the diet. 1 % of McCollum's salt mixture seemed ample, and it was only very recently, when yeast was replaced by vitamins in pure substance, that a slight deficiency of salts seemed possible. The salt mixtures used by all the authors were originally devised for feeding rats and almost certainly contain far more Ca, Na and Cl than is required by insects. It is obvious that, by using a salt mixture which is specific for the requirements of insects, the quantities of salt in the diet could be greatly reduced. Work is in progress to devise such a salt mixture.

(4) From the tests described in this and the following paper it is obvious that cholesterol or another adequate sterol should be added to the diet, unless it is supplied in sufficient quantity with some fat.

SUMMARY

1. The method of breeding six different insect species on a 'synthetic' diet consisting of casein, starch or glucose, cholesterol, yeast, salts and water has been described, and the relative importance of the constituents, carbohydrates, sterols and yeast, has been analysed.

2. If the diet contains water-soluble yeast extract instead of whole yeast, it becomes deficient in sterols and another factor contained in the insoluble fraction of yeast.

3. Some of the insects under investigation require carbohydrates and others not. The presence or absence of carbohydrates is shown to be a determining factor in the distribution of insects on different foods.

4. The composition of the 'synthetic' diet is compared with that of similar diets devised for similar purposes by other authors.

We gratefully acknowledge a Government grant, made by the Royal Society, which, partly, made this investigation possible.

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THE OVIPOSITION RESPONSES OF *CALANDRA GRANARIA* LINN.

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of Zoology, University of Sheffield

(*Received* 1 *May* 1943)

(With Three Text-figures)

his investigation was undertaken following the rvey into the incidence of infestation of stored reals by insects (Munro, 1940), and forms part of a mprehensive research programme initiated by that thor into the problems of the biology and control grain weevils.

Once infestation of a stock of grain has taken place e rate at which it develops depends on the number insects present and on the extent to which the nditions favour their multiplication. Of the factors verning multiplication may be mentioned the rate oviposition, the length of time a female lives before e lays eggs and the length of her ovipository life. hese factors are dependent in turn on the environ- ent, and they have formed the subject of this vestigation with reference to the grain weevil, *alandra granaria*, in stored wheat.

In no earlier work have strictly controlled environ- ental conditions been applied in this connexion. or instance, Kunicke (1936) reports that at 26° C. female lays 200 eggs over an unstated period of me and that at 24° C. adult *Calandra* could live for year. Lavrekhin (1937) states that at 25–27° C. e females lived for about 130 days, and that the erage rate of oviposition was 0·9 egg per day from e 14th to the 54th day, the rate then falling off. nce these results were obtained without any precise ntrol of the atmospheric humidity they are of little lue. With these defects in mind the features of iposition rate, length of preoviposition period and length of ovipository life in *Calandra* have been amined under a series of controlled humidities for ch of several temperatures. In this way some mparison can fairly be made between the effects temperature and atmospheric humidity separately d together.

TECHNIQUE

ft English wheat, previously sterilized at 80° C. r 3 hr., was used throughout the experiments. The ater content of wheat varies considerably (Dendy Elkington, 1920), and to stabilize this factor all ain used was first acclimatized to the atmospheric midity intended to be used in experiment. This as done by exposing thin layers of grain on gauze ays in large desiccators containing appropriate solutions of KOH (Buxton & Mellanby, 1934) for periods of not less than 36 days. The water contents corresponding to the several atmospheric humidities as determined in the wheat by Dr O. W. Richards are given in the following table:

% R.H. of air	Water % in grain
80	15·98
70	13·82
60	13·26
50	11·29
40	10·56

In this way it was ensured that wheat, by virtue of its water content, had no influence on the atmospheric humidity in any experiment, and conversely that the relative humidity of the air effected no change in the water content of the grain.

Temperature control in the chambers used was efficient, thermograph records showing the maintenance of a temperature constant to ±0·2° C.

Humidity of the atmosphere in the vessels was controlled by solutions of KOH, these being made up from data taken from Buxton & Mellanby (1934). Glass jars with cork-seated bakelite covers, capacity 750 c.c., were used, KOH of appropriate specific gravity in egg-cups being placed therein for humidity control.

A typical experiment was set up as follows. A pair of newly emerged weevils, male and female, was placed in each of fifteen gauze-covered tubes, size 3 by ½ in., with ten grains of acclimatized wheat. In each of fifteen other similar tubes a pair of weevils was placed with five grains of wheat. The tubes were then packed into the jar together with the humidity controlling KOH and the jar then screwed up and placed in the temperature chamber in the dark and without any undue mechanical disturbance. In every experiment the weevils used were taken from stocks which had been reared under the same conditions as those about to be applied.

At regular intervals the grain was removed from the tubes for egg counts, the KOH renewed and new grain provided to the weevils after removal of any faecal matter, this having been shown by Dendy

(1920) to be hygroscopic. At 20° C. grain was changed every 3 weeks, at 22·5 and at 25° C. every fortnight, and at 27·5° C. it was renewed every week. By the adoption of a suitable system of numbering the tubes, an estimation of the number of eggs laid by each individual female was possible for each stage throughout her life.

The following table sets out the combined conditions of temperature, humidity and water content of grain employed:

Temp. ...	20° C.	22·5° C.	25° C.	27·5° C.	H_2O content of grain
R.H.	40 %	40 %	40 %	—	10·56
R.H.	50 %	50 %	50 %	50 %	11·29
R.H.	60 %	60 %	60 %	60 %	13·26
R.H.	70 %	70 %	70 %	70 %	13·82
R.H.	—	80 %	80 %	80 %	15·98

As the table shows, the water content of the grain is fixed for each relative humidity. Should this factor prove to be significant in oviposition, therefore, it will be possible to compare its effects against temperature but not against air humidity. In other words food with special reference to its water content represents a third variable which is not fully controlled. The difficulty of effecting a control of this factor is great, since weevils feed preparatory to oviposition (on the same grain on which they oviposit). If, for instance, at any single relative humidity of air, food grain containing a water content out of balance with the air humidity were inserted, no stability of either water content of grain or of air could be maintained.

While the moisture conditions were controlled and recorded in terms of relative humidity, it has to be pointed out that any single relative humidity through a range of temperatures corresponds to different saturation deficiencies of the environmental air, the saturation deficiency increasing with the temperature (Buxton, 1931). Evaporation having been shown to be proportional to saturation deficiency, provided the air is moving at uniform speed (Ramsay, 1935), it is clear that important results are likely to emerge which comparisons between effects of relative humidities at different temperatures will not disclose. For these reasons, the saturation deficiencies in mm. mercury approximately equivalent to the relative humidities and temperatures employed have been indicated in the several figures.

The female perforates the grain with her proboscis, turns round and deposits an egg in the hole so made and then seals the opening with a drop of fluid which sets hard to form an 'egg plug'. Any part of the grain may be selected for oviposition, though there is a strong preference for the junction of the smooth and hairy parts at the end opposite to the embryo (Back & Cotton, 1926). In these experiments eggs were estimated on counts of egg plugs which were visible on the surface of the grain. A more accurate method would have been that recommended by Dr O. W. Richards *in lit*. Here the grain is soaked in 70 % alcohol for 24 hr., the testa is stripped off and the eggs counted from the dissected grain. The concurrent experiments of this investigation made this last method impracticable. A comparison of the two methods reveals that about 8 % of the eggs are overlooked by the first method. It must therefore be recognized that the figures given in this work are inaccurate to this extent, but the general nature of the results given is claimed to present a true picture of the case.

EXPERIMENTAL RESULTS

The results obtained enable us to draw attention to the effects of saturation deficiency (S.D.) of the air and of temperature on the following: (*a*) rate of oviposition, (*b*) total number of eggs laid, (*c*) length of ovipository life, (*d*) total length of life, (*e*) length of preoviposition period; having regard always to the fact that a third variable food is involved, since for each atmospheric humidity the grain used had a particular water content.

There is considerable variation in the performance of individual weevils. All were of similar age at the beginning of an experiment, and the uniformity of conditions applied to separate weevils in any one experiment, together with the fact that all weevils had had applied to them, during their own development, the same conditions as those to which they were subjected in the experiment, forces one to the conclusion that the differences in behaviour mentioned are inherent in the weevils themselves.

This previous acclimatization to the conditions of treatment is important, for it has been shown that previous conditions, different from those of an experiment, can impose on animals a type of behaviour out of relation with the conditions subsequently applied (Mellanby, K., 1939, 1940).

The principal results are summarized in Figs. 1–3, these being arrived at by taking the average of behaviour of ten weevils from each experiment. In every case the results shown are taken from those experiments where a pair of weevils was placed with ten grains of wheat. Similar results, but of a lower order, were obtained in a parallel series of observations in which each pair of weevils was placed with five grains. For reasons of space the results of this latter series have not been recorded.

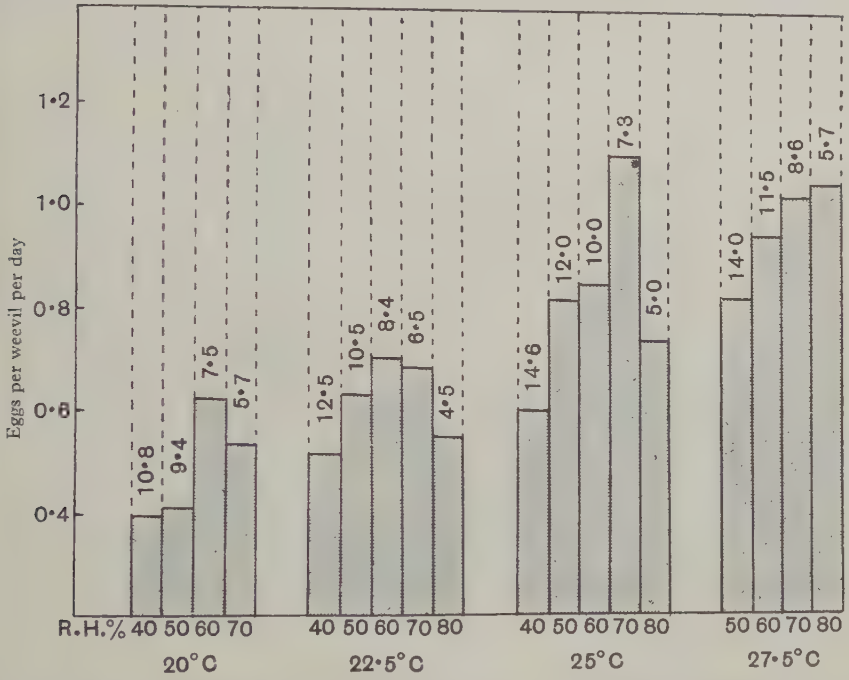
Rate of oviposition

Fig. 1 shows the effects of a range of S.D. at each of four temperatures. The retarding effects of S.D. of increasing scale are obvious. That temperature also is an important factor is shown by comparing equivalent conditions of S.D. at different temperatures. The higher temperature is seen to be correlated with a higher oviposition rate.

For all the temperatures used there appears to be optimum s.d. for rate of oviposition. Thus the llowing combinations of temperature and relative midity are those equivalent to a common s.d. of mm. mercury:

Temp. °C.	Relative humidity %
20	60
23·5	67
25	70
27·5	75

hese are perhaps close enough to the relative midities at which in Fig. 1 the highest ovipository tes are recorded for each temperature, to justify e conclusion that a s.d. *c.* 7·4 mm. mercury re- esents an optimum condition within this range of mperatures (Buxton, 1930). Ramsay (1935) showed at the higher rate of evaporation from an insect at y constant s.d. occurs at higher temperatures than lower because of the increased rate of diffusion at ose higher temperatures. While this is undoubtedly ue it is difficult to attribute the increased rate of iposition to such a cause. It is more likely to be e to an increased rate of metabolism following additional ventilation of the tracheal system which results from a rise in temperature (Wigglesworth, 1939).



g. 1. Oviposition rates (eggs per weevil per day) under controlled conditions of temperature and atmospheric humidity. The numbers below the columns represent the percentage relative humidities of air employed; those above the columns the approximate saturation deficiencies.

In 1937 Lavrekhin examined the fertility of *Calandra*, measuring it in terms of the number of progeny found in grain at the end of a month. His figures are lower than those recorded here, probably because of cannibalism which occurs when two or more larvae compete for life in a single grain and also perhaps because he appears not to have ensured that all weevils were of the same age. He may well have used weevils which had passed their period of most rapid egg production. Lavrekhin further found that the highest fertility at 25° C. occurred after the 14th day and was maintained for a further 35–40 days. All curves (not recorded in this paper for reasons of space) showing rate of oviposition in this work are sigmoid, and the highest rate occurred only after the 30th day in the 25° C. experiments, after which this steady high rate continued for 40 days. There seemed to be no special preference for full as against shrivelled grains for oviposition, though Mathlein (1938) states that weevils will not lay eggs in grain of lower water content than 10%. It is not possible to say how far the latter differed from the driest grains employed by us with 10·56 % water, since there is no information as to Mathlein's method of estimating water content.

Total number of eggs laid (Fig. 2)

Allowing for some unaccountable discrepancies in the results set forth in this figure, it appears that at any given temperature total egg production varies inversely as the saturation deficiency. That this conclusion is valid emerges from a consideration of the effects of a rise in S.D. through a range of temperatures. For example, the columns indicating an experimental condition of 60 % relative humidity at the temperatures from 20 to 27·5° C. demonstrate that the total egg production falls as the S.D. rises in the following order: 7·5, 8·4, 10·0, 11·5 mm. mercury. On the data given it is impossible to estimate accurately the effects of temperature under a condition of constant S.D. By interpolation, however, one can indicate the expected total egg production at such a constant S.D. through the several temperatures. The line *AB* (Fig. 2), for instance, passes through such expected points of egg production at the common S.D. of approximately 11·0 mm. mercury, and it is seen that the egg production goes up as the temperature rises. The same thing is shown by the line *CD* which passes through points of expected egg production at the common S.D. of 8·0 mm. mercury. (A comparison between these two lines enables us to confirm the conclusion already expressed that egg production varies inversely as the S.D.)

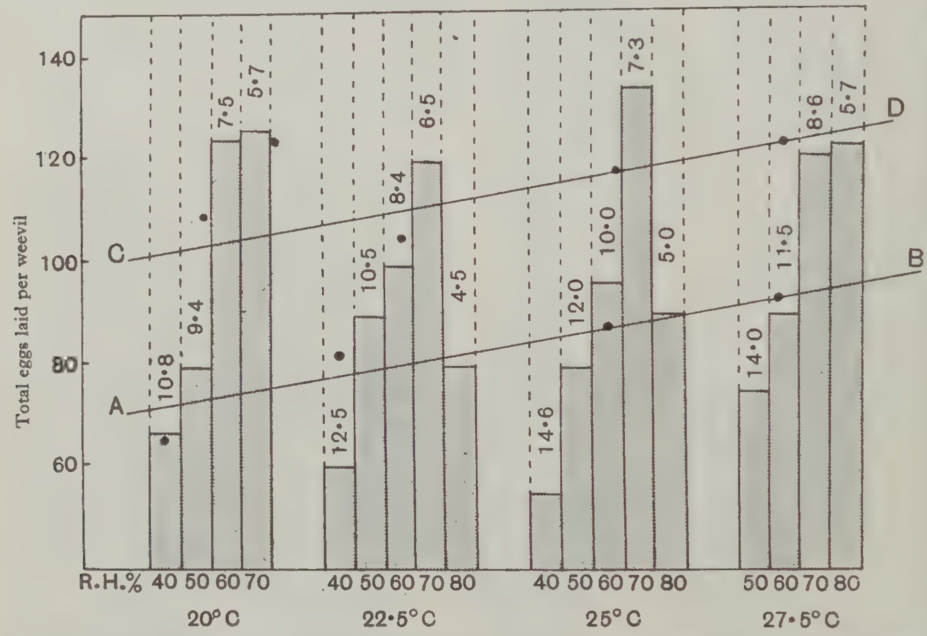


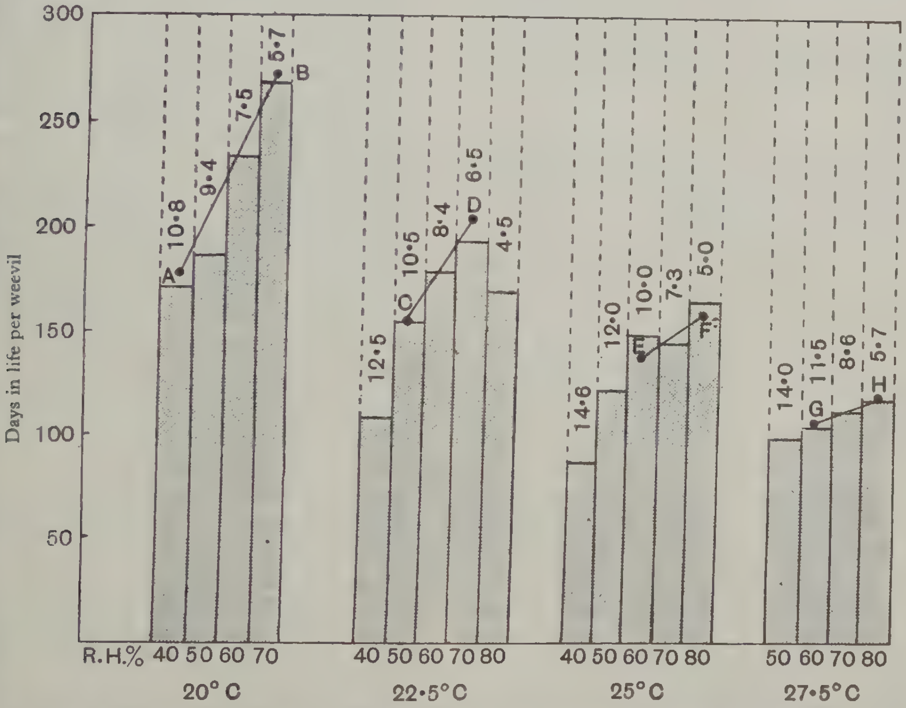
Fig. 2. Total number of eggs laid per weevil during life under controlled conditions of temperature and atmospheric humidity. *AB* passes through points of expected egg production under saturation deficiency of 11·0 mm. mercury. *CD* ditto for saturation deficiency of 8·0 mm. mercury. Numbers above and below the columns as in Fig. 1.

The effect, however, is not solely one of temperature, for by choosing a common S.D. in this way by interpolation, we have passed up the scale of relative humidities from 40 to 60 % and therefore we have at the same time been treating of weevils whose food has varied in its water content, this water content rising from 10·56 % at the lowest temperature to 13·26 % at the highest temperature. How far such differences in water content of food grain are effective in this aspect of weevil behaviour is impossible of exact analysis. In view of the findings of Robinson (1925), who claims that *Calandra* gains weight and thrives more readily on wheat with higher water content, and of Ewer & Ewer (1942), who in *Ptinus* found an increase in egg production when this beetle had access to drinking water, it appears likely that the total egg production in *Calandra* is directly proportional to both temperature and water content of the grain on which this insect feeds.

On a matter of detail Muller's records (1927) fo otal egg production give lower figures than those ndicated here.

Length of life (Fig. 3)

Several conclusions can be drawn from the results mbodied in this figure. For instance, weevils live nger at lower than at higher temperatures. This ct emerges when one views the data as a whole nd more forcibly when comparisons of temperature ffects are made under conditions of S.D. which are pproximately the same. For each temperature it is milarly obvious that length of life varies inversely s the S.D. The quantitative effects of S.D. are not e same, however, at all temperatures. We can, for nstance, by interpolation, indicate the expected ngth of life of weevils under S.D.'s of a uniform nge in the several temperatures. The lines *AB*, *D*, *EF*, and *GH* (Fig. 3) express graphically the pected differences in age of weevils kept under a mmon scale of S.D. ranging from 10·5 to 5·5 mm. ercury at each temperature. As the temperature ses the difference of age of weevils, associated with is constant scale of S.D., becomes less. This unexpected result can be illustrated in another way, viz. by comparing the length of life of weevils under an increasing difference of S.D. through the temperature scale.



ig. 3. Days in life of weevils under controlled conditions of temperature and atmospheric humidity. *AB*, *CD*, *EF* and *GH* represent the expected differences in age of weevils kept within a range of saturation deficiency between 10·5 and 5·5 mm. at each of the temperatures. Numbers above and below the columns as in Fig. 1.

By comparing the length of life of weevils at the several temperatures under the relative humidities of 50 and 70% (Fig. 3), we are in fact examining the age factor under an increasing range of S.D. Table 1 sets out the average longevity in days at each temperature and relative humidity and at each corresponding S.D. In the last two columns of this table are given the differences in age of weevils kept at 50 and 70% relative humidities together with the differences in S.D. associated therewith. The difference in age is greatest where the difference in S.D. is least and vice versa. If an increase in S.D. is the handicap in the life of a terrestrial insect as may logically be assumed, then *a priori* we would expect results of an inverse order to those recorded here, viz. that the greater difference in S.D. should be associated with greater difference in age. We suggest that the solution of this problem lies in the interplay of the two factors, temperature and S.D., in the power of one factor near the optimum to mask the full effects of the other.

The lowest temperature of 20° C. represents (by comparison with the higher temperatures) an adverse condition at which a particular S.D. is able to produce its most marked effect. The higher temperatures, the more favourable as shown by the weevil's behaviour in oviposition rate and total egg production, on the other hand, appear to compensate increasingly for the adverse effects of S.D., especially where this temperature is near the optimum as 27·5° C. appears to be.

It is worth noting that for each of the relative humidities (50 and 70%) under consideration, through the rising scales of S.D. and temperature, there was employed food grain with constant water content.

Table 1. *Differences in age of weevils kept at relative humidities 50 and 70% respectively, compared with reference to the saturation deficiencies which exist at four different temperatures*

Temp. ° C.	Average age in days		S.D. mm.		Difference	
	At 50% R.H.	At 70% R.H.	At 50% R.H.	At 70% R.H.	In age in days	In S.D. mm.
20	190	270	9·4	5·7	80	3·7
22·5	155	195	10·5	6·5	40	4·0
25	125	145	12·0	7·3	20	4·7
27·5	100	110	14·0	8·6	10	5·4

A matter of general interest emerges from a consideration of Figs. 1–3 together. Those conditions of temperature which are conducive to an increased oviposition rate are in general the same as those which lead to shorter life. In view of the not dissimilar performances in total egg production at the several temperatures (Fig. 2) it would seem that weevils have a potential for egg production which is realized in a longer or shorter time according to the temperature applied, provided of course that the conditions are not too severe.

Length of ovipository life

Those conditions appropriate to an increased span of life are such as to allow the animal to continue living after its ovipository powers are spent. Under conditions which shorten life, however, the animal dies when it has ceased to lay eggs. This may be given a different emphasis by assuming that under some conditions the weevil ceases to lay eggs some appreciable time before death, and under others it continues to lay eggs until it dies. At 20 and at 22·5° C. the majority of weevils stop ovipositing about 10–20 days before death while at 25 and 27·5° C. the majority continue to lay eggs until they die. Much variation was noted in these matters, and the results are incapable of any precise analysis.

Preoviposition period

While it must be admitted that the work was not designed to investigate this point fully, such observations as were made are not without interest.

When pupation is ended weevils remain inside the grain husk for a certain length of time. Seldom does emergence from the grain coincide with emergence from the pupal exuvium. Assuming, however, that weevils pass the same length of time between pupation and leaving the grain, the preoviposition period can be measured as the period passed between emergence from the grain and the laying of the first egg. At 20° C. this period is about 21 days, at 22·5° C. about 15 days, at 25° C. 8 days, and at 27·5° C. about 4 days. We may assume from this that the higher temperature has hastened the development of the gonads.

There is nothing to show that air humidity has any marked effect on the duration of this period, though one would expect the water content of the food to produce significant effects. Muller's (1927) claim, that there is a regular preoviposition period of about 3 weeks, provided the temperature is above 12° C., is not substantiated by us. That temperature affects the rate of maturation of gonads in insects has been similarly demonstrated, by Bishopp, Dove & Parman (1915) in the case of the housefly, and by Wille (1920) in *Blatella germanica*. For other references, Uvarov (1931) should be consulted.

DISCUSSION

The environmental factors employed in these experiments, temperature, S.D. and water content of food may be said to act on the animals in two main ways.

The food provides fully or incompletely the necessary material to sustain life and produce eggs. Temperature and air humidity acting through the nervous system affect the general activity of the animal, the first by altering the rate of metabolism, the second by way of the spiracular closing mechanism, imposing on the animal the need under certain circumstances, to conserve water (Mellanby, K., 1934*a*). It is the integration of these factors in the life of weevils which determine their behaviour, setting a limit to the length of life, rate of oviposition and therefore to the total number of eggs produced during a lifetime.

Little work seems to have been done on the oviposition responses of *Calandra*. Such work as exists refers mostly to observations on animals for which not all environmental factors were under experimental control. For instance, Back & Cotton (1926) record, without humidity control, seasonal oviposition activity, taking note of the daily mean temperatures. Lavrekhin (1937) stresses the importance of water content of grain but does not mention any ontrol of air humidity nor the acclimatization of ain to it. In a short review by Richardson (1925) e oviposition responses of insects generally in rela- on to temperature, moisture and nutrition are re- orded, but in the works quoted no single environ- ental factor is treated with. reference to others nder control. For instance, Detouches (1921) notes lengthening of the oviposition period with a reduc- on in temperature, and further finds an increase in e total eggs laid by *Galleria*, but to what extent oisture in the air or in the food were concerned es not emerge.

Within the range of temperatures here used the sults obtained confirm those of earlier workers. ncrease in temperature by raising the rate of meta- olism and hastening the maturation of gonads re- uces the length of the preoviposition period. ishopp *et al.* (1915) agree in this. Also the effect a rise in temperature is to shorten the life and crease the rate of egg production. This in the main onfirms the majority of authors quoted by Uvarov 931), Schubert (1928), for instance, finding a eady increase in the number of eggs laid by *Piesma adratum* in a given time as the temperature rises. hat the higher rate of oviposition due to tempera- re does not always compensate for the shorter life demonstrated by Titschak (1925) in the clothes oth. Disregarding for a moment the important fects of S.D. of air, *Calandra*, under the tempera- res used here, appears to lay about the same umber of eggs at low as at high temperatures, ough the time taken to lay them may vary as widely from 250 days at 20° C. to 100 days at 27·5° C.

It is possible and indeed probable that temperature fects the rate of oviposition or other type of be- aviour for reasons other than those concerned with etabolism, the actual process of laying being de- yed or hastened according as the temperature is w or high. This possibility was examined by Bliss 926), who showed that a temperature applied before viposition (during maturation) is more important in is respect than that applying at the time of ovi- osition. In view of the observations of H. Mellanby 1937) that a temperature of 20° C. (low for the tsetse y) caused pregnant tsetse flies to retain their larvae r periods longer than the normal, the possibility at temperature may have an effect on oviposition irectly must not be overlooked.

Reference has already been made to Ramsay's 1935) interesting experiments where the rate of vaporation, at a constant S.D. of air, is increased as e temperature rises because of changes in diffusion te. Such an effect, however small, would be that f placing increasing difficulties before the animal as e temperature rose. It is not possible to say how r such an environmental factor is of material signi- cance, but it would seem likely from Ramsay's ork that no appreciably harmful effect would arise ntil temperatures higher than those employed here ad been used.

Turning to the effects of atmospheric moisture on these aspects of behaviour in *Calandra*, it is clear that increasing S.D. of the air puts a brake on the animal's egg-laying activities. The higher the S.D., the lower the oviposition rate, the fewer the eggs laid and the shorter the life that the insect enjoys provided the temperature is constant. In other words, within the ranges of temperature and S.D. employed these two environmental factors are antagonistic. But while temperature would appear to exert its effects metabolically, with concomitant effects on the oviposition responses of the animal, S.D. seems not to operate in this way.

That humidity of the air has no effect on metabolic rate in insects has been shown by a number of workers (Buxton & Lewis, 1934; Mellanby, K., 1932, 1934*c*, 1935 *a*, *b*; Gunn & Cosway, 1942). It would seem therefore that where an insect lives for a shorter time at a higher S.D. than at a lower, the shorter life is not attributable to changes in metabolic rate. Factors other than temperature are known to be concerned in the determination of thermal death-points, i.e. longevity of insects (Mellanby, K., 1934*b*). Thus starved *Culex* cannot survive in temperatures as high as those which have been fed. Further, the thermal death-point is higher in atmospheres of low S.D. than where the air is drier. Here a high S.D. is a limiting factor.

There is some evidence for the existence of an optimum S.D. at each temperature for oviposition rate. It is not possible to point to any such relation between S.D. and longevity. Both a higher temperature and a higher S.D. are conducive to shortening the span of life, but whereas the former acts so as to enable a full potential of egg production to be realized in a minimum time, the latter reduces the span of life, inhibiting at the same time the full expression of oviposition. This inhibition is almost certainly due to the animal's reaction of closing its spiracles so as to reduce water loss, by which action the effects of higher temperatures towards increased ventilation of the spiracles are countered. This interplay of temperature and S.D. has already received comment with reference to length of life (Fig. 3). That this same interplay is effective in oviposition rates and in relation to the total number of eggs laid, emerges in a similar manner from a consideration of Figs. 1 and 2.

Though the chief controlled factors in these experiments have been temperature and S.D., the third, factor of water content of the grain, imperfectly controlled for reasons already given, cannot be ignored. Any condition of food which renders it less acceptable and less easily masticated and digested must serve as a deterrent to egg production. Such a condition is low water content. On the other hand, food containing more water is softer, and easier of mastication and digestion. The weevil in respect of the types of behaviour here dealt with cannot escape the impact of this part of its environment. It is claimed therefore that for length of life, rate of oviposition and number of eggs laid, the low figures associated with the greater S.D.'s are also in part attributable to low water content of grain, though this effect is incapable of quantitative analysis.

When researchers have examined the lengths of time which insects can survive under certain applied conditions they have very commonly eliminated the food factor by working on starving animals. In whatever manner the conditions have affected the animal's ability to survive in such experiments, the fact remains that in many cases death was due to starvation following either the exhaustion of food reserves or the inability to use those reserves as the other conditions of life became severe. Though here the food factor is not possible of precise analysis the conclusion can safely be drawn that when weevils die under the more severe temperature or S.D. conditions, starvation is not the cause of death, though malnutrition owing to inadequate water in the food is a contributory factor. Where starvation conditions apply, any conditions conducive to long life are advantageous. But, as in this work, where a female with access to food lays roughly the same number of eggs whether the temperature be 20 or 27·5° C., but lays them at different rates, there seems to be no reason for assuming that a weevil gains any special advantage from either a long life or a short one.

From the economic point of view, however, a short life with full ovipository capacity leads to more rapid infestation of grain stocks, and the conditions which determine these two results are clearly of the greatest economic importance. They lead to rapid increase in numbers and are therefore advantageous to the species. They are the conditions which should be avoided in the storage of grain if the attempts to keep the pest to harmless proportions are to meet with success.

SUMMARY

1. The oviposition responses of *Calandra granaria* as manifested by the rate of oviposition, total eggs laid, length of life, length of ovipository life and length of preoviposition period, have been investigated under controlled conditions of temperature and saturation deficiency of air, grain having been acclimatized in its water content to the relative humidity of the air.

2. *Calandra* lives for a shorter time under high than under low temperatures but lays eggs at a greater rate, thus compensating for the shorter life.

3. There is evidence for the existence of an optimum saturation deficiency at each temperature for oviposition rate.

4. Weevils are shorter lived at high saturation deficiencies than they are at low.

5. The total number of eggs laid by weevils is smaller at high than at low saturation deficiencies of air.

6. Water content of the food grain contributes to these results in that dry food is conducive to low rate of oviposition, low total egg production and shorter life.

The work was made possible by a grant from the Royal Society to whom our thanks are accorded. We wish also to thank Dr O. W. Richards for help in matters of technique, for estimating the water content of samples of the grain used, and for testing the validity of the method employed to estimate the number of eggs laid in the grain.

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ON THE BEHAVIOUR OF WIREWORMS OF THE GENUS *AGRIOTES* ESCH. (COLEOPTERA, ELATERIDAE)

I. REACTIONS TO HUMIDITY

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(With Six Text-figures)

umidity reactions have recently been demonrated in a number of terrestrial arthropods. In veral instances—for example in *Locusta migratoria* Kennedy, 1937), *Culex fatigans* (Thomson, 1938), *latta orientalis* (Gunn & Cosway, 1938), *Tenebrio olitor* (Pielou & Gunn, 1940) and in *Pediculus manus corporis* (Wigglesworth, 1941)—it has been own that such responses lead to the avoidance of oist air, a type of behaviour which is not easily sociated with any specific physiological requireents. On the other hand, certain animals such as oodlice which are known to possess little capacity resist desiccation, avoid dry, and aggregate in umid air (Gunn, 1937; Waloff, 1941); this type behaviour is clearly of some adaptive significance.

Wireworms also are unable to withstand prolonged esiccation. Thus according to the data of Subklew 934*a*), large larvae weighing about 50 mg. lose o to half their body weight in 3 days when introuced into still, half-saturated air at 20° C. With this mind, the humidity behaviour was tested in an ternative chamber. When it became clear that an tense response was shown to certain humidity adients, the nature of the humidity receptors and e mechanisms displayed in orientating to humidity ere also examined. The present paper deals with ese aspects of the humidity behaviour and their lation to the humidity conditions prevailing in the ormal environment, the soil.

METHODS AND MATERIAL

he apparatus used for testing the general humidity ehaviour of wireworms was substantially the same the 'alternative chamber' described by Gunn & ennedy (1936). It consisted of a circular glass essel, 22 cm. in diameter and 6 cm. deep, which as closed by an airtight glass roof (Fig. 1 A). In der to reduce slightly the area of contact between e two halves of the chamber, a vertical partition (*a*), cm. deep, was attached to the roof. The desired umidities were maintained by means of sulphuric cid-water mixtures (Wilson, 1921) contained in ishes (*b*) on the floor. The arena was provided by circular tray of wire gauze (*c*), supported from the floor, and attached to the sides of the chamber by a ring of plasticine.

If wireworms are not provided with some form of contact, their movements may be abnormally rapid and sustained, and under these conditions the sharpness of the reactions at certain steep humidity gradients is considerably reduced; in order to remedy this defect, several layers of glass beads (average diameter about 0·25 cm.) were introduced into the gauze tray (*d*). This proved to be a very satisfactory means of 'damping' their movements; wireworms placed on top of the beads immediately burrowed into them, and then crawled for the most part between the gauze and the beads.

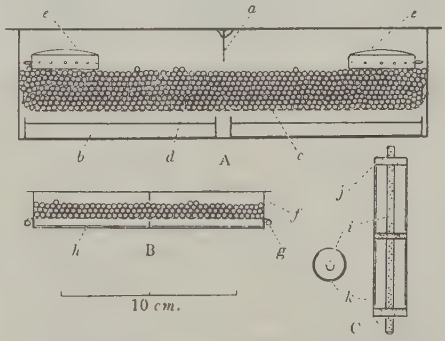

Fig. 1. Apparatus used for investigating the humidity behaviour of *Agriotes*. A, large, and B, small alternative chamber in section; C, plan and section of alternative 'burrow'. Further explanation in text.

The humidities on the two sides of the chamber were recorded by means of Edney paper hygrometers (*e*) placed on top of the beads.

As it was impossible to view the woreworms conveniently when they were crawling below the beads, the procedure adopted was to count the numbers of larvae on either side at the end of the experiment. Usually twenty-five larvae were used simultaneously in the chamber. If, however, the larvae were admitted through a hole in the roof after the humidity gradient had become established, they never became properly dispersed and usually remained in a cluster in the centre of the arena. To avoid this difficulty, the larvae were scattered in the 'middle zone' (this was a narrow region extending across the centre of the chamber and corresponding to the steepest part of the gradient; animals recorded from this zone could not properly be assigned to either side); the lid was then replaced and the gradient was allowed to develop with the larvae inside. Controls showed that no appreciable bias was involved in this procedure. With a humidity range within the chamber of 90–100% R.H. the length of each experiment was 6 hr.; with lower humidities, 3 hr. This represented a compromise between the effects of the inactivity of the larvae at high humidities and the activity at lower humidities; the latter sometimes led to the clustering of the larvae if the experiment was too protracted. If such an aggregation did occur the experiment was rejected. The different lengths of the experiments are unlikely to have any effect on the intensity of the reaction for, once dispersal has been achieved, there is evidently an equilibrium between the numbers on the two sides which is maintained by the humidity reaction: increasing the length of the experiment will not materially affect this relationship. The humidity gradient was usually fully established within 2 hr.; it may therefore be assumed that the larvae were in fact reacting to the final humidities recorded by the hygrometers.

As an estimate of the intensity of the reaction the excess percentage ratio $100\left(\frac{W-D}{W+D}\right)$ (Gunn & Cosway, 1938) was employed. In this expression W and D are the numbers of animals in the 'wet' and 'dry' sides respectively. The W/D ratio (Gunn, 1937) has sometimes been calculated for purposes of comparison. In the first expression the theoretical value for no reaction is 0·0%, in the latter 1·0. The small numbers of animals recorded from the middle zone are omitted from the calculations. Each experiment was repeated eight times; altogether, therefore, 200 position records were obtained for each pair of humidities tested. Controls in which the humidity of the chamber was constant throughout were kept for each experiment.

For the analysis of the mechanisms of the humidity reaction single larvae were observed in a smaller alternative chamber (Fig. 1 B). This consisted of a glass ring (f) made from a ground-down Petri dish; it was covered by a glass lid and stood on a false floor of muslin stretched between a wire ring (g) (Wigglesworth, 1941). The required humidities were maintained by means of filter papers soaked in various saturated salt solutions (Spencer, 1926); these filter papers (h) were placed below the muslin arena in the cover of a Petri dish selected as having the same diameter as the glass ring. The muslin was covered by only three layers of beads and the apparatus was illuminated from above. Tracks of individual larvae were recorded by tracing them in ink on the lid; these were subsequently transferred to paper.

In addition, observations were made on single wireworms crawling in 'burrows' (Fig. 1 C) constructed in the following manner. Hollow straws of uniform diameter were split in two longitudinally, and the channels thus formed were closed by strips of perforated cellophane (i) which were cemented to the cut edges of the straw. Each 'burrow' could be passed through holes in three waxed corks (j) which formed equidistant partitions in a glass tube. Each half of the burrow was then surrounded by a more or less isolated jacket into which strips of filter paper (k), soaked in the required salt solutions, could be introduced. A wireworm could then be inserted into one of the open ends of the burrow and its behaviour noted as it crossed the steep humidity gradient into the opposite side.

The wireworms used in these studies were collected in batches in the field, and were then kept in jars of moist sand provided with potato slices until required for experiment. The most abundant species in these collections were *Agriotes obscurus* L. and *A. lineatus* L., and these formed the material for this research; the larvae of these species cannot be separated on morphological grounds; they were distinguished from the closely related species *A. sputator* L. by the diagnostic features given by Guéniat (1934). Larvae ranging in length from 5 to 20 mm. were tested for their humidity behaviour, but no essential differences were noted between the various size categories which must in fact have included several instars. In practice, wireworms about 10–15 mm. in length were most commonly used.

RESULTS

The direction, range and intensity of the reaction

The results of six typical experiments are shown in Table 1. The average mean excess percentage of all the controls was 1·8%, which closely approximated the theoretical value for no reaction. Twice the standard deviation was 12·2%; intensities greater than this can then be taken as indicating a significant reaction.

An inspection of Table 1 shows that under certain conditions *Agriotes* reacts intensely to humidity; for example, with alternative humidities of 99·5 and 92% R.H., 190 larvae were recovered from the wet side and none from the dry side; the remaining ten position records were from the middle zone. This complete avoidance of the dry side gives an excess percentage on the wet side of 100%, and a W/D ratio of infinity. Smaller humidity differences within approximately the same range yield decreasing intensities of reaction, but the response is still apparent with differences too minute for the hygrometers to record accurately: thus a significant response was obtained with humidities of about 100 and 99·5 % R.H. (the equilibrium humidities of the controlling solutions were in this case 99 and 100 % R.H.). These results are shown diagrammatically in Fig. 2.

It is also clear that within this small humidity range (90–100 % R.H.), the intensity depends on the available humidity differences and not on any particular humidity within this range. Thus, in an example included in Table 1, the excess percentage on the wet side with alternatives of 100 and 98·5 % R.H. was 63 %; a further experiment where alternative humidities of 97 and 95 % R.H. were used, also yielded a reaction intensity of 63 %.

Table 1. *Results obtained from six experiments at 17° C.*

Relative humidities in the chamber (% R.H.):						
(*a*) Highest	99·5	100	97	89	79	64
(*b*) Lowest	92	98·5	95	82	71·5	54
(*c*) Difference (*a*−*b*)	7·5	1·5	2	7	7·5	10
Number of position records in each zone:						
Moister (*W*)	190	151	147	169	129	87
Drier (*D*)	0	34	34	18	54	86
Middle	10	15	19	13	17	27
Intensities of reaction:						
(I) As ratio *W*/*D*	∞	4·4	4·3	9·4	2·4	1·0
(II) As excess percentage	100	63	63	81	41	1

On the other hand, when larvae are offered the choice of a series of humidities differing in range but not in the humidity differences available, it is immediately apparent that the intensity of the reaction becomes progressively reduced as the highest humidity in the chamber is lowered. Thus with a 7·5 % R.H. difference between alternative humidities both of which lay within the range 80–90 % R.H., the excess percentage on the wet side was 81 %. The intensity for the same humidity difference within the range 70–80 % R.H. was 41 %, while within the range 60–70 % R.H., and at all lower humidities, no significant reaction at all was recorded.

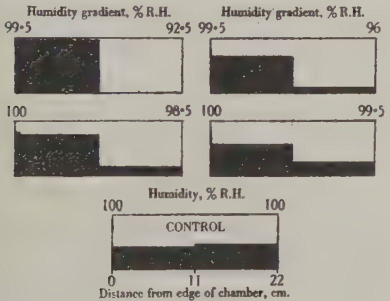

Fig. 2. Relative distribution of wireworms in the alternative chamber after exposure to alternative humidities lying within the range 90–100 % R.H.

As the intensity of the response is evidently influenced by the range as well as by the differences in humidity which are available, it is impossible to group all the points on a single curve. In Fig. 3, which includes all the data derived from mass experiments in the alternative chamber at 17° C., definite ranges of humidity (100–90, 90–80 % R.H., etc.) have been arbitrarily selected; within these narrow limits the reaction intensity appears to be approximately proportionate to the difference in humidity. The reason for the marked decline in the intensity of the reaction at low humidities will be discussed later.

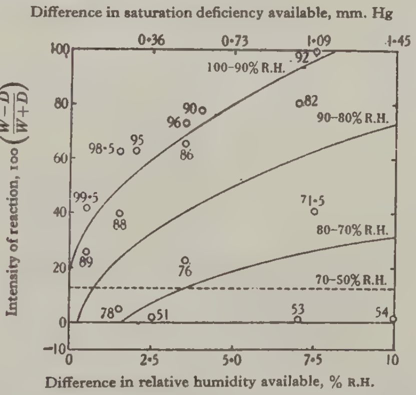

Fig. 3. The intensity and range of the humidity reaction at 17° C. The figure by each point records the lower humidity in the chamber in that experiment; the unbroken lines separate experiments carried out within different arbitrarily selected humidity ranges (100–90 % R.H. etc.); the broken line is drawn at twice the standard deviation of the controls. Note the high reaction intensities when the alternative humidities are close to saturation and the complete disappearance of the reaction at lower humidities.

The results of an experiment by Subklew (1934*b*) are in agreement with the behaviour described above. This author placed a number of wireworms in an empty tube in which a humidity gradient was maintained by means of moist cotton-wool at one end and a drying agent at the other. After an interval it was found that all the larvae had aggregated at the humid end of the tube.

The effect of temperature on the reaction

The intensity of the humidity reaction at different temperatures has been investigated in *Culex fatigans* (Thomson, 1938) and in *Tenebrio molitor* (Pielou & Gunn, 1940). In both cases it was found that the reaction intensity was more closely related to the relative humidity than to the saturation deficiency, or evaporating power, of the air.

The behaviour of *Agriotes* was tested at three temperatures, 11, 17 and 25° C., using the alternative chamber in the same manner as previously. Sulphuric acid solutions maintaining alternative relative humidities of 99·5 and 92% R.H., 99·5 and 96% R.H., and 100 and 98·5% R.H. were employed. The hygrometer readings showed that the difference between any of the three pairs of alternative humidities was the same at all three temperatures: on the other hand, the difference in saturation deficiency was of course greatest at the highest temperature.

In Fig. 4 the excess percentages given by these experiments are plotted against the available differences in relative humidity (A), and against the calculated differences in saturation deficiency (B). It will be clear that the scatter is less when saturation deficiency is used as the measure of humidity. These observations are then consistent with the view that the intensity of the humidity reaction in *Agriotes* is dependent on the evaporation of water from some site on the body; the attempts to localize this region are described in the next section.

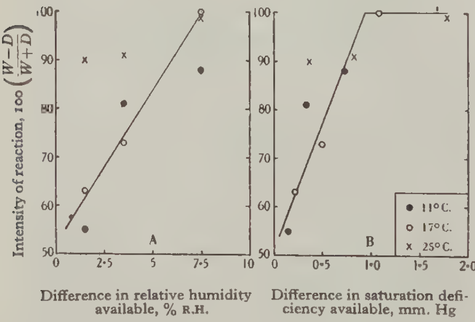

Fig. 4. The intensity of the reaction at different temperatures and humidities. The higher humidity of each pair of alternatives used was nearly 100% R.H. In A, reaction intensities are plotted against the available differences in relative humidity, and in B, against the differences in saturation deficiency.

It should be noted that the activity of the larvae was not noticeably reduced at 11° C.; low reaction intensities at this temperature cannot then be due to the inactivity of the wireworms.

The humidity 'receptors'

In finding the position of the humidity receptors, advantage was taken of the intense reaction shown by larvae to alternative humidities of 92 and 99·5% R.H. In a preliminary series of experiments the pits on the 9th abdominal segment were covered with shellac; twenty-five larvae with the pits thus covered were used simultaneously in the alternative chamber. 100 position records obtained with these wireworms gave an excess percentage on the wet side of 94%. From this it would appear that these structures, for which various possible functions have been proposed (Langenbuch, 1932), are not concerned with the response to humidity.

Further possible sites of the receptors include some or all of the head appendages (antennae, maxillary and labial palps), or the whole integumental surface. These possibilities were tested with two batches of twenty-five larvae; in one batch the abdomen of each larva was smeared with vaseline; in the other, the head and mouthparts were similarly treated. This did not noticeably interfere with normal locomotion, for the elongated shape of the larva made possible the thorough covering of the abdomen, comprising the greater part of the body surface, without entangling the legs. Both batches were then tested four times in the alternative chamber.

The larvae with the abdomen covered gave an excess percentage on the wet side of 83%, while those with the head covered yielded a reaction intensity of only 22%. The response then, if not eliminated, was at least considerably reduced by greasing the mouthparts, while covering the greater part of the integumental surface had comparatively little effect. These results seemed to indicate that the receptors lay on the head, and that loss of water from the whole integument was not an essential factor in the evocation of the reaction.

In order to localize the receptors more precisely, groups of wireworms, deprived of some or all of their head appendages, were tested in the same humidity gradient. It has been recognized that amputation may affect the behaviour in other ways than by removing the specific receptors which are being tested (Pielou, 1940). Thus, the removal of the antenna (Wigglesworth & Gillett, 1934) may give rise to a state of lowered activity which will naturally reduce the intensity of the response to a particular stimulus. In *Agriotes* apparently normal activity was retained, even after the removal of all the head appendages. In addition, such operations may impair the general health of the insect. In this respect it may perhaps be mentioned that wireworms deprived of all the appendages survived in a healthy state for several months; in the course of the experiments one larva moulted and regenerated the lost organs.

The relevant mouthparts of *Agriotes* are shown in Fig. 5. The basal components of the maxilla and labium are partially fused, and form a single functional unit which can be protracted and retracted by muscles inserted on the dorsal surface. The labial palps are borne by the stout ligula. The fleshy, two-segmented galeae arise near the insertion of the maxillary palps, while the lacinia is plate-like and is closely associated with the 'brush'. The diminutive antennae are also two-segmented structures.

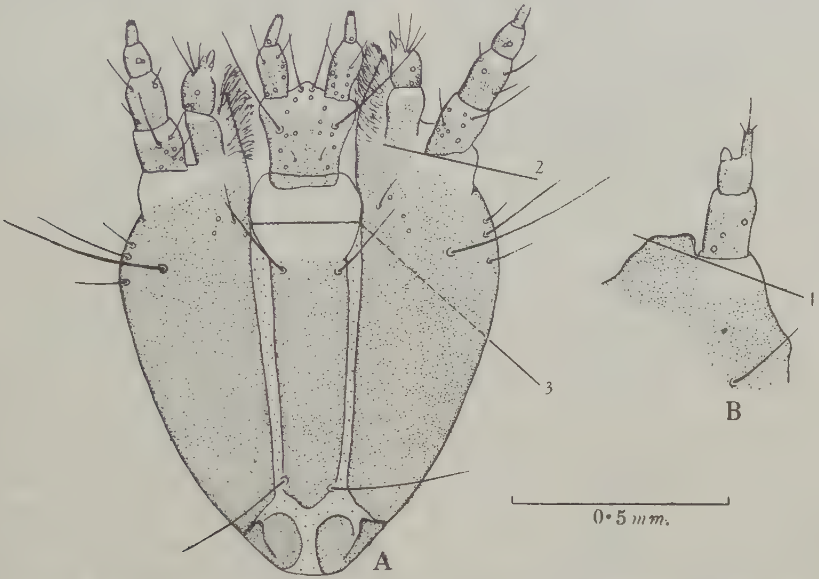

Fig. 5. The head appendages concerned with the response to humidity in *Agriotes*. A, maxilla and labium; B, antenna. The figures indicate the sites of the various amputations. Note the campaniform organs (shown as circles) and the unsclerotized regions.

Table 2. *The intensity of the humidity reaction in larvae with various combinations of bilaterally amputated appendages*

Organs amputated		None	
Excess percentage		100	
Organs amputated	Antennae	Maxillary palps	Labial palps
Excess percentage	79 ± 4·2	92 ± 2·3	90 ± 3·0
Organs amputated	Antennae and maxillary palps	Antennae and labial palps	Maxillary and labial palps
Excess percentage	38 ± 6·0	65 ± 4·1	91 ± 3·1
Organs amputated		Antennae, maxillary and labial palps	
Excess percentage		−4 ± 12·1	

The removal of whole organs was accomplished by inserting a finely pointed needle through the basal intersegmental membrane. Before the operation the wireworms were lightly etherized. Whole organs, which were removed bilaterally, were amputated in the following combinations, each in batches of twenty-five larvae: (1) antennae, (2) maxillary palps, (3) labial palps, (4) antennae and maxillary palps, (5) antennae and labial palps, (6) maxillary and labial palps, and (7) antennae, maxillary and labial palps. For the exact sites of the operations reference may be made to Fig. 5; in all cases the galea was removed with the maxillary palps and the ligula with the labial palps. After a few days had been allowed for recovery, each batch was tested eight times in the 92–99·5 % R.H. gradient at 17° C., so that 200 position records were obtained from larvae of each category. The results are shown in Table 2, where reaction intensities are again expressed as excess percentages on the wet side.

An inspection of Table 2 shows that the bilateral amputation of either antennae, maxillary palps or labial palps only reduces the intensity by a small fraction; no one pair of these organs is then solely concerned with the response. Removal of the antennae has more effect in reducing the intensity than the removal of the other appendages. On the whole, however, in each of these cases avoidance of the lower humidity is clearly shown; indeed, it appears to be almost uninfluenced by the absence of maxillary or labial palps. If two pairs of appendages are removed, the intensity of the reaction diminishes more markedly: thus when the antennae alone were left intact there was still a very intense response (91 %), but when, on the other hand, the maxillary palps and the labial palps were the sole remaining organs, excess percentages of only 65 and 38 were recorded. These experiments show that each of the three pairs of appendages is concerned with the humidity reaction, though not to the same extent. Finally, when all the organs are amputated simultaneously the reaction is entirely eliminated (excess percentage of -4). Evidently then these appendages either bear the only sensilla concerned with the humidity reaction, or in themselves constitute the sole 'receptors'.

The temperature experiments have shown that the intensity of the reaction can be more closely related to the saturation deficiency of the air than to its relative humidity: that is to say, the reaction probably depends on water loss rather than on any hygrometer mechanism. If this is so, and the site of evaporation is on the head appendages, it might be possible to correlate the surface areas exposed by the appendages with their respective contributions to the reaction intensity. The maxillary palps and antennae are, in fact, roughly equal in volume and surface area, and both are considerably larger than the labial palps. This can perhaps be partly correlated with the present results, for the labial palps alone give a much less intense response than the maxillary palps or antennae alone. On the other hand, the relatively greater importance of the antennae than the maxillary palps cannot be explained along these lines.

In order to see whether this hypothesis could be reconciled with their morphology, the mouthparts were examined in whole mounts and sections, as well as in the living animal under a binocular microscope. In the living animal the antennae, maxillary and labial palps present a rather uniform appearance: each is made up of a small number of annuli of relatively thin integument separated from one another by areas of colourless membrane. Particularly conspicuous is the broad cushion of unsclerotized cuticle on which the base of each appendage is inserted. It is possible that these regions in particular may be associated with rapid water loss. In life the haemolymph in these organs is kept under a slight positive pressure by the contraction of the thoracic musculature, and amputation is usually accompanied by a gush of blood; in the anaesthetized animal when the musculature is relaxed this occurs more rarely.

While it is not intended to describe in detail the sensilla on the head appendages, a brief consideration of the main types and their distribution is necessary in order that they may be evaluated as possible hygro-receptors. The sensilla are as follows: (*a*) *Campaniform organs*. These are distributed on the dorsal surfaces of the antenna, galea, ligula, maxillary and labial palps; a few are found more basally (Fig. 5). In unstained whole mounts these sensilla appear as conspicuous circular areas, and were described by Hawkins (1936) as 'round pit-like sensory pores'. (*b*) *Peg organs*. A few of these sensilla, seldom more than a dozen, occur on the ventral surfaces of the labial and maxillary palps and the galea; they are absent from the antennae. (*c*) *Tactile bristles*. These are found on all the head appendages; they are for the most part thick-walled although their structure is somewhat variable. Certain other structures, possibly sensory in function, include the following: (*d*) the numerous minute projections borne by the distal segments of the labial and maxillary palps, and (*e*) the cup-shaped and apparently perforate structure carried by the distal joint of the antenna.

It will be noted that apart from thick-walled bristles, the only organs found on antennae, maxillary and labial palps are the campaniform sensilla, and these are unlikely to respond to any other than mechanical stimuli (Pringle, 1938). In view of the probable importance of the peg organs as hygro-receptors in *Tenebrio* (Pielou, 1940), it is of interest that in *Agriotes* these sensilla cannot function in a similar manner, for, as has been shown above, there is still an intense residual response when only the antennae, which do not bear peg organs, are left intact. Since, also, (*d*) is confined to the maxillary and labial palps, and (*e*) to the antenna, these structures may also be ruled out as possible humidity receptors.

The uniformity in the appearance and structure of the head appendages as well as the negative evidence of the lack of any specific sensilla, lends support to the hypothesis that the loss of water from the head appendages initiates the humidity reaction. The sites of evaporation cannot be precisely delimited, but they are likely to include the whole surface of the appendages and perhaps, in particular, the unsclerotized intersegmental regions.

The mechanisms of orientation

It has been shown that wireworms avoid dry, and aggregate in moist air, but it remains to decide the nature of orientation mechanisms which enable this result to be achieved. This was investigated by observing single animals in the small alternative chamber described above. Four typical tracks are reproduced in Fig. 6: each 30 sec. interval is indicated by a cross-bar on the track; definite halts for longer periods are shown by numbers which record the half-minutes which elapsed before locomotion began again.

If larvae are exposed to uniformly saturated air their movements are usually slow and spasmodic (Fig. 6 A); there may be frequent halts, often of considerable duration, when the larva is immobile save for slight movements of the head. If, on the other hand, the humidity within the chamber is lower than 100% R.H., the rate of locomotion is greatly increased, and the animals rarely, if ever, become inactive. There is no evidence of adaptation to dry air; on the contrary, in unsaturated atmospheres larvae remain in a state of violent activity until they die from desiccation. At all humidities the rate of change of direction appears to be entirely at random. This type of reaction which involves a differential rate of movement or a difference in the amount of activity (number of 'rests', etc.) when the animal is exposed to two intensities of a stimulus, has been termed an ortho-kinesis (Gunn *et al.* 1937): this mechanism forms an integral part of the humidity behaviour of several arthropods—for instance of *Tenebrio* (Gunn & Pielou, 1940) and *Porcellio* (Gunn, 1937).

Ortho-kinesis can also be demonstrated in *Agriotes* if alternative humidities are used in the chamber, the higher humidity being saturated air and the alternative some lower humidity. If, as occasionally happens, a larva crosses the humidity gradient from the moist side of the chamber to the dry, the rate of movement, as may be judged from an inspection of the tracks, instantly increases (Fig. 6 B). In the type of apparatus used here the animal is then rapidly brought back to the moister half of the chamber. On re-entering saturated air, the rate of locomotion usually decreases within a few minutes, the change in rate, however, seeming to be less rapid than when the larva emerges from moist into dry air.

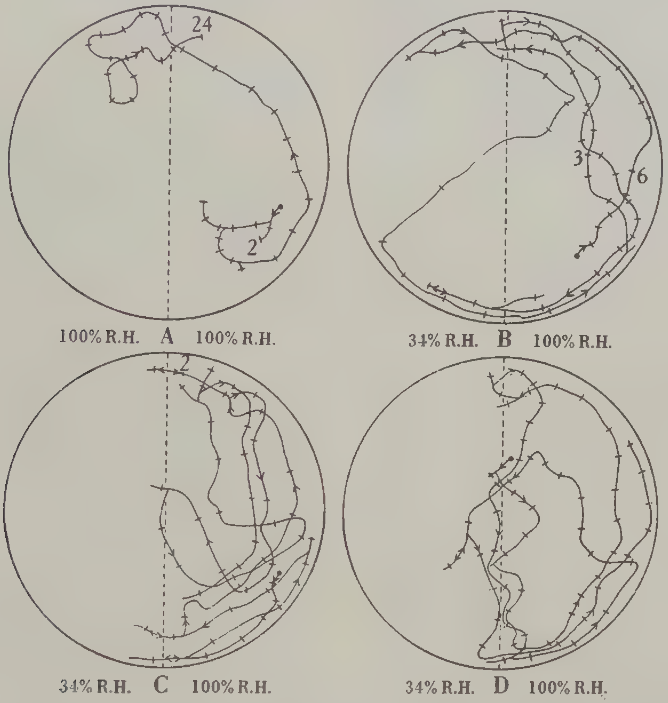

Fig. 6. Tracks followed by single wireworms in the small alternative chamber; each track was completed in 30 min.; successive half-minute intervals are indicated by cross-bars. A, behaviour in uniformly saturated air; B–D, examples of the mechanisms of orientation to dry air; in B, the activity is seen to be greater on the dry side; in B, C and D, several instances of directed reactions are shown; the larva enters the dry half of the arena, hesitates, and then recoils backwards into the wet half.

There is also a further very typical orientation mechanism which is usually clearly exhibited if the alternative humidity to saturated air is rather low (e.g. 34% R.H., a saturated solution of $MgCl_2$ being used). On approaching the humidity boundary the larva hesitates, moves its head from side to side, and finally recoils backwards into the moist side. Fig. 6 C, D shows several examples of this type of behaviour; the larva has sometimes made short excursions into the lower humidity but has then invariably retreated along the same track, afterwards moving off in a different direction. This striking reaction can be seen to even better advantage in the artificial 'burrow' described on p. 44. If a wireworm, selected so that its diameter is just accommodated by the burrow, is inserted into the moist half, it will gradually move up to the junction with the dry half; it then stops, manipulates its head appendages, and then retreats backwards into the humid section of the burrow. The distance of recoil may be very considerable, and wireworms frequently ejected themselves with some violence from the end of the tube (a distance of 10 cm. in the burrows used). This peculiar type of locomotion, no doubt a characteristic response to unfavourable stimuli in general, has frequently been observed in wireworms moving about in self-constructed burrows in sand or soil, particularly if they had migrated into a region where the medium had obviously become rather dry. The recoil was usually sufficient to carry the larvae out of this region.

There is no evidence that the turning reactions of *Agriotes* are affected by humidity, nor indeed would this be expected in view of the normal habitat of the larva. This mechanism is not then an example of klino-kinesis even in its extreme form (see Gunn & Walshe, 1941; Wigglesworth, 1941). The orientation is in fact a directed one, the interesting feature in this case being that under natural conditions it is necessarily so since the direction of movement is restricted by the limits of the burrow. Two types of directed orientation (taxes) have been distinguished. In the first place, an animal behaving klino-tactically 'compares' in successive intervals of time the intensity of the stimulus to which it is reacting; secondly, an animal exhibiting tropo-taxis compares the two intensities of the stimulus simultaneously by means of bilaterally placed sense organs (Fraenkel & Gunn, 1940). As a mechanism of reacting to the diffuse stimulus of humidity, tropo-taxis is at least theoretically possible although it has not yet been demonstrated in any insect. One means of distinguishing between these two mechanisms is to eliminate the receptors on one side; many animals behaving tropo-tactically will then show circus movements.

In order to examine this point, the head appendages which, as has been shown above, constitute the humidity 'receptors' were eliminated on one side. In a batch of twenty-five larvae the left antenna and left maxillary palp were amputated. These wireworms were tested individually in the small alternative chamber and showed no tendency to perform circus movements. They were also subjected to mass experiments in the large alternative chamber where alternative humidities of 92 and 99% R.H. were maintained. The unilateral removal of the receptors had no effect on the intensity of the reaction, for out of a total of seventy-five position records seventy-one were from the moist side while only one larva was recovered from the dry side. Had circus movements been involved they would probably have been revealed by an increased number of animals on the dry side. It may be concluded therefore that this orientation is not tropo-tactic but is probably klino-tactic. It may also be pointed out in this connexion that in *Agriotes*, as in all wireworms, the head appendages are extremely short, and it is difficult to believe that they could be affected bilaterally, but differentially, by any gradient of humidity however steep.

This analysis of the mechanisms of orientation throws some light on the results obtained from the mass experiments in the large alternative chamber, although it is unfortunately impossible to decide with any precision the relative contribution made by each type of mechanism to the general humidity behaviour. One of the most striking features of these results was the very complete reaction at the extreme moist end of the humidity range (100% of the larvae avoiding 92% R.H. and aggregating in 99·5% R.H.). In the light of the mechanisms described above it seems evident that the clear-cut separation brought about by these humidities is to a great extent caused by the operation of the klino-tactic mechanism. This reaction is no doubt very effective in causing the avoidance of the lower humidity if the larva happens to cross the boundary from the high into the low humidity. At the same time it seems probable that the low hygro-kinesis which results in the partial immobilization of the larvae on the moist side ('virtual immobility'; Gunn & Pielou, 1940) is a very important contributory factor, and this was certainly the impression gained when the larvae were being removed from the moist half of the chamber at the end of an experiment.

Fig. 3 also indicates, however, that as the humidity range in the alternative chamber is lowered, the intensity of the response to similar differences in humidity declines, until within the general humidity range of 50–70% R.H., for instance, there is no significant reaction to differences as large as 15% R.H. The reason for this is probably quite simple. At low humidities the larvae are always intensely active, and as a result, those which are crossing the arena are rapidly carried over the steepest part of the humidity gradient into the lower, though relatively uniform, humidity in the opposite side of the chamber. This has the effect of virtually eliminating the avoiding mechanism which is so well shown at higher humidities. It may be added as a corollary that the rate of movement is an important factor in the normal operation of this klino-tactic mechanism. Lack of contact, which also leads to an increased rate of movement, exercises a similar inhibitory effect on the reaction: but the complete disappearance of the reaction at low humidities must also indicate that the rate of movement is not in any simple way proportionate to the relative humidity or saturation deficiency; that is to say, it is probable that the differences in the rates of movement between larvae in uniform humidities of 100 and 90% R.H. is much greater than that between humidities of for instance, 60 and 70% R.H. This point has not been examined experimentally.

DISCUSSION

It has been shown that wireworms react strongly to certain gradients of humidity. If some allowance is made for the slightly different form and mode of operation of the apparatus used to test this behaviour, it would appear that *Agriotes* avoids dry air with an intensity roughly equal to the intensity of avoidance of moist air by *Tenebrio* (Pielou & Gunn, 1940) or by *Culex* (Thomson, 1938). Over the range of humidities where the intense reaction is given, differences in saturation deficiency of 1 mm. Hg, or in relative humidity of 7·5% R.H. at 17° C. over a 22 cm. gradient, are sufficient to bring about a successful avoiding reaction by nearly every individual, while statistically significant reactions are given by much smaller humidity differences. But in *Agriotes* as in *Tenebrio* and *Culex* the intense reaction only operates over a small part of the whole humidity range: in the first insect, if one alternative is saturated air, the reaction leads to the avoidance of any humidity which falls short of saturation, while in *Tenebrio*, *Culex* and *Pediculus* (Wigglesworth, 1941), only high humidities are avoided, the insects appearing more or less indifferent to lower humidities. In a sense it would seem that *Agriotes* also is indifferent to large humidity differences if the values for the alternative humidities are under 70% R.H. It has been suggested, however, that the failure of the reaction is due rather to the elimination of an integral part of the orientation mechanism under unfavourable physiological conditions rather than to any real 'indifference' to humidity as a factor capable of influencing behaviour. In some respects the general behaviour of *Agriotes* most closely resembles that of *Porcellio*. Thus Gunn (1937) has shown that woodlice avoid dry air; at the same time his work indicated that the region of maximum sensitivity to small humidity differences lay in the region of 50% R.H. Waloff (1941) has, however, demonstrated that, under certain conditions at least, woodlice exhibit an intense reaction at the extreme moist end of the humidity scale, for instance, with alternatives of 86 and 100% R.H.

An interesting feature of the humidity reactions of terrestrial arthropods is their association with two fundamentally different types of receptor apparatus. These might be described as 'hygrometer' and 'evaporimeter' receptors respectively. The first depends on the presence of specific sensilla capable of functioning hygrometrically; the second type of 'receptor' appears to depend in the first instance on the evaporation of water either from a localized region of the body or from the whole integument. Apart from verifying the presence or absence of the relevant sensilla, the only experimental method available for deciding between these alternatives involves the examination of the reaction intensity at different temperatures and humidities—a method which assumes that the intensity remains approximately the same at the various uniform temperatures employed. In *Agriotes*, experiments at three temperatures have indicated that the intensity of the reaction is related more closely to the differences in saturation deficiency than to the differences in relative humidity offered in the alternative chamber. This is suggestive of the 'evaporimeter' receptor. On the other hand, similar experiments have shown that in *Culex* (Thomson, 1938) and *Tenebrio* (Pielou & Gunn, 1940) the reverse is true.

With regard to the actual 'receptors', it appears that in *Agriotes* the response is dependent on the integrity of the head appendages, and it has proved impossible to associate it with any particular type of sensillum. In *Porcellio*, Gunn was also unable to localize the position of the hygro-receptors, and Waloff has suggested that loss of water from the integument and the consequent concentration of the body fluids may initiate the reaction. The latter author also proposes an alternative explanation, namely, that a secondary proprioceptive mechanism may be involved. Although in *Agriotes* all the head appendages concerned with the reaction are well supplied with campaniform sensilla, it is difficult to believe that evaporation could stimulate these organs in any way, for even extreme desiccation causes no visible shrinkage. The rapidity with which the reaction develops suggests that local changes in osmotic pressure may perhaps be sufficient to stimulate the peripheral sensory nerves directly.

This type of 'evaporimeter' receptor contrasts with the 'hygrometer' receptor occurring, for example, in *Tenebrio* (Pielou, 1940), where the numerous peg and pit peg organs are the sensilla concerned with the response. In mosquitoes a similar function has been proposed for the fine hairs of the antenna (Necheles, 1925), while in *Pediculus* the reaction has been shown to depend on the presence of the 'tuft organs'—sensilla whose structure also suggests that they may function hygroscopically (Wigglesworth, 1941).

It may be noted that while the 'hygrometer' receptor might conceivably be associated with reactions in either direction, the 'evaporimeter' type is only likely to function successfully if, by the operation of the receptor, dry air is avoided. It is not surprising then that *Tenebrio*, *Culex* and *Pediculus*, all of which avoid moist air, appear to possess the former type of receptor.

The nature of the humidity receptors may also influence the mechanisms displayed in orientating to humidity. Thus in klino-kinesis, which involves the random turning movements of the animal, sensory adaptation is an essential adjunct; but, as Waloff (1941) has pointed out, adaptation to a low humidity is unlikely to take place in an animal which is consistently losing water, or from which appropriate sensilla are absent. In *Agriotes* klino-kinesis is in fact absent, although the burrowing habit of the larva would perhaps exclude this mechanism in any case. In *Porcellio* also typical klino-kinesis seems to be lacking. This is in direct contrast to the importance of this mechanism in the humidity behaviour of *Tenebrio* (Gunn & Pielou, 1940) and *Pediculus* (Wigglesworth, 1941).

If, as seems probable, the humidity responses of *Agriotes* are set in train by the evaporation of water from the head appendages, some suggestions can be offered as to the factors leading to the operation of the orientation mechanisms. It has been noted above that wireworms in unsaturated air are always active. It seems reasonable to suppose that this kinesis is maintained by a relatively constant rate of evaporation from the appendages. A larva crossing a humidity gradient into drier air will, on the other hand, be subjected to a rapid increase in the rate of water loss. An abrupt change of this sort may serve to evoke the second type of mechanism described, namely, the sudden recoil from dry air. As, however, desiccation has no effect on the intensity of the response, both mechanisms probably depend on the rates of evaporation rather than on the level of concentration, whether local or general, of the body fluid.

The recoiling mechanism may be classified as a klino-taxis for it seems to involve a comparison of intensities (or rates of evaporation) at successive intervals of time; also, the head appendages constitute collectively what is in effect a single receptor. At the same time, the response is more simple than that shown by, for instance, a maggot orientating to light—a typical example of klino-taxis (Fraenkel & Gunn, 1940)—where, in order to achieve orientation, a succession of such comparisons is made. In *Agriotes* the whole reaction comes into play after a certain threshold of stimulation is exceeded. For this mechanism the term 'shock reaction' (Mast, 1938), if unsatisfactory in some respects, is certainly apt.

Humidity behaviour and the normal environment

The inability of wireworms to withstand desiccation has already been mentioned. This has been shown by Subklew (1934*a*) to be due to the exceptional permeability of the cuticle to water. The rapid passage of water-soluble arsenicals through the integument (Woodworth, 1938) also points to the same conclusion.

Now according to the recent data of Evans (1943), the osmotic pressure of the haemolymph of *Agriotes* is approximately equivalent to that of a 0·3 M sucrose solution. As the vapour-pressure depression of such a solution is 0·6% and the relative humidity about 99·4% R.H., wireworms exposed to humidities lower than this will lose water by evaporation, and—what is important in this context—the rate of water loss through the integument will be relatively high in comparison with the rate of loss in insects possessing less permeable cuticles.

It is necessary therefore to inquire whether there are reasons for doubting the common assumption that the soil atmosphere is under all conditions uniformly saturated. If so, the risk of desiccation may be real, and the humidity behaviour may have an important, if indirect, bearing on the maintenance of the normal water balance. Actually, substances in solution reduce the relative humidity to some slight extent. Of greater importance, however, are the hygroscopic properties of soils. Thus, for the wireworm, a critical state will be reached when the soil moisture corresponds to a vapour-pressure depression of 0·6%, a value equivalent to a 'suction' pressure on the *pF* scale of 3·9 (see Evans, 1943). Such conditions are of course most likely to occur during the summer drying out of the surface layers of the soil.

The absorbtion isotherms of the various types of soils given by Puri, Crowther & Keen (1925) are instructive from several points of view. These curves show the typical S-shape displayed by many hygroscopic materials; they indicate that if a soil is dried, comparatively large amounts of water have to be removed to shift the equilibrium humidity from, for instance, 99 to 90% R.H., while with further water loss, small differences in moisture content cause a rapid drop in humidity. It follows that the saturation deficiencies which may be expected to occur most frequently, during, for example, periods when the soil is moderately dry, will be small and therefore difficult to detect experimentally. The values given by Puri *et al.* leave little doubt, however, that such deficiencies must often occur, although their incidence will clearly depend, among other factors, on climatic conditions and the soil type. It is of interest to note in this connexion that Lebedeff (1928), using a specially designed hair hygrometer, was able to demonstrate in the field that high ground temperatures were usually accompanied by measurable saturation deficiencies, although this was only true of the surface 10 cm. of soil.

The humidity behaviour of wireworms is evidently correlated with these environmental conditions. The 'preferendum' is 100% R.H., for in the presence of saturated air all other humidities are avoided; but it is also significant that humidities close to saturation, such as are expected to prevail in the soil, represent just that range over which the reactions are most sensitive; indeed, the whole mechanism of the response is only capable of functioning successfully in the presence of a high humidity.

It cannot, of course, be stated with certainty that humidity is a limiting environmental factor, but the numerous references in the literature to the migration of wireworms out of the surface layers of the soil during periods of drought (e.g. Miwa & Yanagihara, 1929) are perhaps suggestive of this; doubtless other factors, including the effects of soil moisture (which are discussed in the next paper (Lees, 1943)), contribute to this behaviour, but it is probable that the response to humidity plays an integral part.

SUMMARY

1. The general humidity behaviour, the humidity receptors and the orientation mechanisms have been studied in the larvae of *Agriotes*.

2. Wireworms avoid dry air, the intensity of avoidance being greatest when the alternatives are close to saturation. Within this humidity range a difference of 7·5% R.H. in the alternative chamber (at 17° C.) is sufficient to ensure the successful avoidance of the lower humidity by nearly every individual, while statistically significant reactions are obtained with differences as small as 0·5% R.H. At lower ranges of humidity the same differences yield progressively less intense reactions; at low humidities the response is entirely eliminated.

3. The intensity of the reaction is in better accord with the humidity differences when these are expressed as saturation deficiencies rather than as relative humidities. This suggests that the reaction is initiated by the evaporation of water ('evaporimeter' receptor) and not by the operation of receptors which function hygrometrically ('hygrometer' receptors).

4. The humidity 'receptors' lie on the head. Amputation experiments indicate that the relevant sites of evaporation are distributed between the antennae, maxillary and labial palps; the structure of these appendages is compatible with such a function; on the other hand, sensilla which might conceivably have a hygroscopic function are absent.

5. Orientation is achieved by the operation of two mechanisms. First, larvae are more active in dry than in moist air (low hygro-kinesis); secondly, larvae show a directed response to low humidities (klino-taxis); this is displayed as a powerful backward recoil if the wireworm crosses a steep gradient from moist into dry air. No tropo-tactic component is involved. The failure of the reaction at low humidities is explained by the great activity of the larvae which interferes with the operation of the klino-tactic mechanism. It is suggested that the kinesis is maintained by a relatively constant rate of water loss from the head appendages, while the klino-taxis is initiated by any sudden increase in the rate of evaporation.

6. The behaviour can readily be correlated with the humidity conditions prevailing in the soil; the necessity for such a sensitive response is possibly dictated by the permeability of the cuticle which renders wireworms peculiarly liable to water loss in unsaturated atmospheres.

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ON THE BEHAVIOUR OF WIREWORMS OF THE GENUS *AGRIOTES* ESCH. (COLEOPTERA, ELATERIDAE)

II. REACTIONS TO MOISTURE

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(With Plate 1 and One Text-figure)

The influence of water, as distinct from humidity, on insect behaviour has not been widely investigated, but with regard to the special problem of wireworm distribution, soil moisture has been recognized as an environmental factor of considerable importance (Thomas, 1940).

Most of the information on this subject relates to observations made in the field, but several authors have carried out experiments in which wireworms were offered the choice of soils of different moisture content. Thus Langenbuch (1932) showed that there was a tendency for larvae to aggregate in wet soils; Subklew (1934*a*) recorded such aggregations from soils of the highest water-holding capacity; Campbell (1937) emphasized the rapidity with which wireworms migrate out of dry soils and also tested the responses to gravity. These experiments have been repeated here using a modified technique.

Despite the economic importance of wireworms little attention has been given to their feeding reactions or to the effect of moisture on these responses. Ecological data again provide most of the relevant information. A method has, however, been devised by Langenbuch (1932) for examining the feeding reactions; in this, a number of larvae are confined in vessels containing sand or soil of the required moisture, each vessel also being provided with a potato slice; the feeding activity can then be judged roughly by eye. In the present paper this type of experiment has been somewhat extended.

METHODS

In experiments on moisture preference most authors have used some form of multiple alternative chamber. Usually the apparatus has been a simple tube with perforated partitions separating the various moistures; the wireworms are either placed in the middle section or a number are placed in each section, their distribution being recorded at the end of the experiment.

This type of apparatus is subject to several disadvantages. In the first place the two end compartments are in a unique position. Then, as often constructed, the chamber is so long that unless the experiment is unduly protracted an equilibrium distribution is not attained; owing to the number of alternatives often offered at the same time, the results may be too complicated to lend themselves to any simple interpretation. There is also a further very real objection to the use of tubular chambers, for when they are being loaded it is almost inevitable that the larvae will be inserted when the tube is held vertically, even if the final position is to be horizontal. Now wireworms placed on a soil surface immediately begin to burrow downwards, and once covered by the soil there is a tendency for burrowing to continue in the same direction. Hence, unless the tubes are loaded very rapidly, aggregation will occur at the end of the tube which was underneath when loading was carried out.

These objections have been met by using choice chambers which could be loaded horizontally. These were rectangular glass troughs measuring 20 × 4 × 4 cm.; the lids were held in place by elastic bands. After horizontal loading, such a chamber could either be left in a horizontal position or stood vertically, and in this way the gravity responses could also be tested. Only two moistures were used simultaneously in a trough, the mode of operation thus being somewhat similar to that of the alternative humidity chamber devised by Gunn & Kennedy (1936). In using these alternative moisture chambers, sand or soil of the required moisture was loaded into the trough, the two moistures being kept separate during loading by a thin metal partition. Fifty larvae were placed in the middle of one alternative section and the partition was removed. After 24 hr. the numbers of wireworms in the two sections were counted. Over this period there was comparatively little diffusion of water even when extreme alternatives were used. Usually four chambers were used simultaneously; in two of these, wireworms were placed in each of the two alternatives; the other two chambers contained uniform moistures throughout and served as controls.

In the present work seived and washed sand with a grain size of 0·05–0·1 mm. was almost exclusively used as a medium. Its use facilitated drying and the subsequent addition of the required quantities of water; moreover, it was of course certain that all the available moisture was freely contained in pore spaces of reasonably constant dimensions. Since the percentage of hygroscopic water in sand is negligible, even quite low moistures maintain an equilibrium humidity of 100% R.H., and any reaction recorded is to moisture and not to humidity.

Moistures are throughout expressed as percentages of saturation; the value for saturation was obtained by adding water to the sand in a measuring cylinder until free water just appeared at the surface.

An apparatus in which the behaviour of individual wireworms could be watched was also extensively used. This consisted simply of two 50 × 20 cm. rectangular sheets of plate glass separated by a rubber gasket. The space between the plates could be filled with a given quantity of sand at the required moisture which was evenly spread by rolling (Pl. 1, fig. 1).

In contrast with the uniformity of their humidity behaviour, different collections of *Agriotes* sometimes differed slightly in their responses to moisture. Each batch has therefore been maintained and numbered separately (e.g. lot 13, etc.).

RESULTS

Observations on the burrowing of wireworms

Although not of immediate importance to the present account certain observations on the manner of construction of the burrow may conveniently be included here.

The most effective burrowing movements in rather loose sand or soil are made by flexing the abdomen and thrusting the head forward. Sometimes the insect picks up individual grains of sand with the mandibles and deposits them at the side of the burrow.

The establishment and extension of burrows by single wireworms was observed in the plate apparatus. In one example a wireworm made the following daily additions to the length of the burrow—43, 58, 21, 12 and 0 cm.; after the fourth day and until the tenth there was no further extension of the system, the wireworm spending most of the time moving about in the already formed burrow. It is characteristic of the burrow pattern that it is usually made up of intersections of three tracks; this probably indicates that on reaching an old burrow the larva prefers to enter it rather than to construct a new burrow across it. It seems probable then that a wireworm burrow is at least a semi-permanent structure, although extensions of the ramifying system may occur at intervals.

From this point of view the observations of Tenhet & Howe (1939) on the burrowing of the sand wireworm *Horistonotus uhlerii* are of interest. These authors found that the larvae exhibited a distinct preference for moving through established burrow systems rather than constructing new ones: movement through such a system might be extremely rapid and was for the most part vertical in direction.

Reactions to alternative moistures

The effect of moisture on the behaviour of *Agriotes* is best illustrated by an example in which rather extreme moistures are employed; the response is then sufficiently intense to be immediately apparent.

Most information is available concerning the migration between moisture alternatives of 10 and 65% saturation. In this series of experiments in which some 500 wireworms were used, four alternative chambers were run simultaneously; in two of these, fifty wireworms were placed in the 10 and 65% sections respectively, and were allowed to migrate into the other alternative; the other pair also contained fifty wireworms each but sand of only one saturation (10 or 65%). In addition, there was a similar series with the chambers vertical instead of horizontal. Each experiment was repeated four times with this batch of larvae (lots 14–15) so that 200 position records were provided by larvae reacting to each set of conditions. Another batch of larvae with a different history (lot 13) was also tested in a similar manner, but the results which differed in some respects from those considered here, are more conveniently treated separately. The length of each experiment was 24 hr. and the temperature 17° C.

The average distributions of the larvae at the end of the experiments are shown in Table 1 and Text-fig. 1. An inspection of Table 1 at once indicates that many more larvae burrow from 10 into 65% (71%) than from 65 into 10% (19%), and this is true of both horizontal and vertical series. Such a distribution might be brought about in two ways; either the moisture content of the sand influences the rate of burrowing, or some avoiding mechanism is called into play as the larvae enter the dry section. The controls with uniform moistures show that the former possibility is correct, since, for example, in the horizontal series the same percentage migrate from 65 into 65% as from 65 into 10% (19%).

Certain of these mean values have been compared for significance by submitting them to a series of *t*-tests; the results are shown in the first column at the base of Table 1. This comparison shows that the differences between the numbers migrating from 10 to 65% and from 65 to 10% are clearly significant. It may also be noted that the numbers moving from 10 to 65% are considerably and significantly greater than in the controls with a uniform moisture of 10%. The probable explanation of this fact is that a proportion of the larvae (about $71 - 51 = 20\%$) moving from 10 into 10% have by the end of the experiment migrated back into the initial section. On the other hand, few or none which have reached 65 from 10% migrate back into 10%.

The wireworms of lots 14–15 were used in these experiments within a week of their collection in the field. It subsequently appeared that they were considerably more active than the larvae of another, apparently healthy batch which had been kept in a constant temperature room at 17° C. for some 6 months. An exactly comparable series of experiments were carried out with this material (lot 13); these results are also included in Table 1.

Table 1. *The migration of wireworms (lots 14–15 and 13) between alternative moistures of 10 and 65% saturation. The moistures in which the wireworms were placed initially are shown in heavy type*

Horizontal series	Alternative moistures	**10**|65	**10**|10	**65**|10	**65**|65
	Percentage migrating: (a) Lots 14–15	71	51	19	19
	Percentage migrating: (b) Lot 13	38	21	5	12
Vertical series	Alternative moistures	$\frac{\mathbf{10}}{65}$	$\frac{\mathbf{10}}{10}$	$\frac{10}{\mathbf{65}}$	$\frac{65}{\mathbf{65}}$
	Percentage migrating: (a) Lots 14–15	78	51	7	11
	Percentage migrating: (b) Lot 13	48	27	2	5

Statistical analysis of Table 1 (data of lots 14–15 only)

Comparison of	*P*
10|65 with **65**|10	< 0·01
10|65 with **10**|10	0·01
65|65 with **65**|10	> 0·1

Comparison of	*P*
10|65 with $\frac{\mathbf{10}}{65}$	> 0·1
10|10 with $\frac{\mathbf{10}}{10}$	> 0·1
65|10 with $\frac{10}{\mathbf{65}}$	0·1
65|65 with $\frac{65}{\mathbf{65}}$	> 0·1

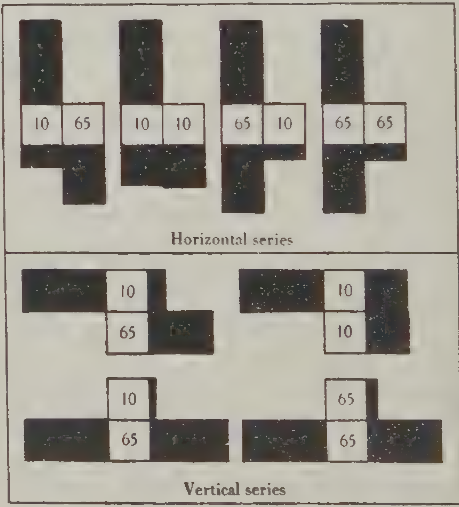
It is evident that the *relative* intensity of the reaction to alternative moistures in lot 13 wireworms was very similar to that shown by lots 14–15; for example, in the former, 38% migrated from 10 to 65% and 5% from 65 into 10%, while in the latter active sample corresponding values of 71 and 19% were recorded. Probably therefore, the only difference in the behaviour of these two collections of larvae lay in their activity; this is also borne out by the parallelism of the other values.

Other similar experiments using alternative moistures of 10 and 30% and 30 and 65% were also carried out, but apart from the fact that the response was less intense, the results did not differ in any respect from those reported above. As in the 10/65% series, more wireworms moved into the wet from the dry sections than vice versa.

The moisture reaction and gravity

The series of experiments summarized in Table 1 and Text-fig. 1 should also provide information about the gravity responses of *Agriotes* if these in fact exist. The relevant mean values are compared for significance in the second column at the base of Table 1.

The percentages of wireworms burrowing from 10 to 65% were very similar whether the troughs were vertical or horizontal. Similarly, in the controls with a uniform moisture of 10% the numbers moving downwards or sideways were identical (both 51%). On the other hand, in those experiments where the vertical trough contained a moisture of 65% at the base, rather fewer wireworms moved upwards than in the comparable horizontal series



Text-fig. 1. Relative distribution of wireworms in the alternative chamber at the beginning and end of four experiments in which alternative moistures of 10 and 65% saturation were provided. The results of the four controls with uniform moistures are also shown.

(7 and 11 % against 19 and 19 %). These differences, although not statistically significant, may reflect a real reaction, but one which has no connexion with any gravity response. In the vertical troughs it is of course impossible to prevent the gradual gravitation of water from the upper into the lower sections. This will have the effect of still further immobilizing the larvae, and therefore the small percentages of wireworms moving out from these very moist sections are perhaps only to be expected. From these results one may then tentatively conclude that burrowing larvae are unaffected by gravity.

The effect of moisture on the burrowing activity of single larvae

An attempt was made to investigate the burrowing activity of single larvae confined in the plate apparatus, but although it was possible to standardize the compaction of the sand to some extent, the effect of moisture on the cohesion of the sand particles could not be allowed for. Possibly for this reason, considerable irregularities were noted in the results which will not be reported in detail.

In general, however, it may be stated that low moistures favoured intense burrowing activity while high moistures frequently resulted in the more or less complete immobilization of the larvae. In a single example, one wireworm tested fifteen times in moistures of 10, 30 and 65 % saturation, burrowed distances of 6·6 ± 1·6, 2·2 ± 0·8 and 1·2 ± 0·5 cm./hr. respectively.

The behaviour of wireworms crossing a steep gradient of moisture was also tested. Such a gradient was set up by loading equal quantities of 10 and 65 % saturated sand into the cavity between the plates, the moistures being separated during loading by a narrow glass rod. Larvae burrowing from the moist into the dry sand showed no tendency to avoid the lower moisture, nor were the turning reactions affected. It is evident therefore that the mechanism of the reaction to moisture involves only the relative rates of movement (ortho-kinesis: Gunn, Kennedy & Pielou, 1937). These results are in agreement with the main conclusions derived from the analysis of the mass experiments.

The influence of moisture on the feeding reactions

In preliminary investigations by Dr E. T. Burtt it was observed that wireworms placed in tubes of wet sand containing potato slices did not readily attack the food. Pl. 1, fig. 2 shows a series of such slices which had been placed in the centres of 200 c.c. honey jars containing sand at saturations ranging from 5 to 85 %. Each vessel, except in the control series, also contained ten larvae. The slices, which were removed after 24 hr., evidently indicate that the feeding reaction is most intense at low moistures; very little feeding takes place at the higher saturations.

It is desirable to estimate the intensity of the feeding reaction in a more quantitative manner than is possible by mere inspection of the slices. Unfortunately, as such slices very rapidly imbibe water in wet sand and lose it in dry, an estimation of the amount eaten by weighing, even if controls are kept, is impracticable. Two other quantitative methods were therefore used.* In the first, circles of black-surfaced paper saturated either with water or carrot juice were inserted at the base of honey jars each of which also contained sand of the desired moisture content and ten medium sized wireworms. After 24 hr. the papers were removed. The marks left by the mandibles on the papers clearly indicated the extent of the feeding activity; the relative intensity of this activity could be estimated either by counting the number of bites on the papers or by measuring the area bitten; at high moistures the bites are usually too indefinite to be easily counted so that the second alternative was adopted, the areas bitten being expressed in terms of arbitrary units of a squared micrometer eyepiece.

A typical series of feeding papers is shown in Pl. 1, fig. 3. The results of nine such experiments are reported in Table 2. Exps. 11–13 were carried out on successive days, the same set of wireworms

Table 2. *The effect of the moisture content of the medium (sand) on the areas of feeding papers showing wireworm bites. Areas, in arbitrary units.*

Index nos. of experiments	Papers soaked in carrot juice					Papers soaked in water				
	% saturation of sand					% saturation of sand				
	10	30	45	65	80	10	30	45	65	80
11–13	322	373	233	143	80	1	5	2	0	0
14–16	563	457	205	222	129	0	0	1	2	0
17–19	1053	439	405	233	65	11	0	0	0	0
Mean	646	423	281	199	91	4	2	1	1	0

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* The use of baited papers for testing the gustatory responses of wireworms originated with Dr W. H. Thorpe of this Department. In selecting wheat grains as suitable food for quantitative estimations, I have followed the unpublished results of Mr D. S. Falconer.

being used at the same moistures. Afterwards all the larvae were returned to sand at 30% saturation. Nos. 14–16 were carried out one week, and 17–19 two weeks later, the same procedure being followed as with the first series. From the consecutive daily experiments any cumulative effect which moisture might have upon the feeding reactions should have been apparent. As no such effect was noted, the results of consecutive experiments have been summated.

The data in Table 2 show that the response is primarily a feeding reaction and not a reaction to moisture for the control papers soaked in water show extremely little biting even at low moistures. During the period in which the behaviour was tested there was a considerable increase in the feeding activity. The explanation of this trend is not clear, but it is possible that the majority of the larvae were passing from the non-feeding to the feeding phase of the inter-moult period (Evans & Gough, 1942). Whatever the explanation, it is evident that there is always a conspicuously greater amount of biting at low than at high moistures; at the same time, the effect of moisture is gradual, the intensity of the feeding reaction being approximately related to the absolute water content of the medium.

Table 3. *The number of wheat grains attacked, and the amount eaten by wireworms in sand of varying moisture content. Ten tubes were used at each moisture and each contained a single grain and a single wireworm*

% saturation of sand	5	10	30	45	65	80
	Number of grains attacked					
Small tubes, 24 hr.	8	6	4	4	2	4
Small tubes, 72 hr.	10	7	6	6	5	4
Large tubes, 24 hr.	9	5	4	3	1	1
Large tubes, 72 hr.	10	8	4	4	2	3
	Amount eaten, mg. per wireworm					
Small tubes, 24 hr.	—	8·5	5·5	5·0	4·0	10·5
Small tubes, 72 hr.	—	17·1	19·0	22·2	15·2	13·7
Large tubes, 24 hr.	—	10·6	8·2	8·0	4·5	5·0
Large tubes, 72 hr.	—	14·0	18·5	14·5	21·5	15·0
Mean variation of control grains, mg.	—	−0·3	−0·1	+0·3	+0·6	−0·1
σ	—	2·0	1·7	2·0	2·2	2·0

It is possible to investigate the effect of moisture more fully by the use of a second method designed to test the feeding reactions of single wireworms. In this, soaked and killed wheat grains are utilized as food for the larvae. Such grains are readily attacked; moreover their weight provides a reliable indication of the amount eaten, for the fluctuations in weight on introduction into dry or wet sand are slight and random.

The results obtained from the mass experiments on the feeding reaction may be due to the operation of at least two types of response. In the first place, since it has been shown that moisture has a considerable effect on the burrowing activity, it is possible that a large proportion of the wireworms never reach the food in very moist sand. Secondly, even if the larva reaches the food, the rate of feeding may be affected by the moisture content of the medium. In order to test this point, experiments were carried out in which single larvae and wheat grains were used; ten tubes, each containing one wireworm and one grain, were kept at six different moistures. Two sizes of tube (3 × 1 in. and 2 × ½ in.) were employed. The grains were finally removed and weighed after 24 or 72 hr. In this manner forty feeding records were obtained at each moisture; forty control tubes containing grains but no larvae were also kept.

The results are given in Table 3. When one considers the number of grains attacked at the various moistures, it is apparent, naturally enough, that more grains are attacked in the 72 than in the 24 hr. experiments; but it is also true that in each series more grains are attacked at low than at high moistures. If this is because the wireworms, owing to their greater activity, are better able to reach the grains in tubes containing relatively dry sand, then this disparity should be less in the small tubes where the distance the wireworm has to burrow before finding the grain is comparatively small. This expectation is partially fulfilled for in the 24 hr. series the numbers of grains attacked in the small tubes are 8, 6, 4, 4, 2, 4 (8 in the driest and 4 in the wettest), while the comparable figures for the large tubes are 9, 5, 4, 3, 1, 1. The same trend may also be noted in the 72 hr. experiments. In spite of this effect, however, it is obvious that there is still a greater tendency for the larvae to attack the food if the environment is, in general, a dry one.

On the other hand, the examination of the amount of wheat grain eaten at the various moistures shows that there is no clear-cut relationship between these two variables (Table 3). It would seem therefore that if a larva does attack a wheat grain the rate of feeding may be approximately the same whether the grain is in sand with a high or a low moisture content. From these results one may conclude that at least part of the apparent effect of diminished feeding activity at high moistures, observable in the mass experiments, is in fact due to the decreased locomotor activity of the larvae. It is impossible, however, to consider burrowing and feeding activity as two entirely separate reactions, for in many cases wireworms in wet sand could be observed lying directly adjacent to the food without attempting to attack it. Indeed the dissociation of these two responses is in some ways undesirable, for both are dependent on muscular activity, and it is just this which seems to be inhibited by excess moisture.

DISCUSSION

The choice of alternative moistures by wireworms has been studied by Langenbuch (1932), Subklew (1934*a*) and Campbell (1937). All these authors used multiple alternative chambers of cylindrical shape. Langenbuch observed that whether the wettest sections were at the top or bottom, they always contained the largest numbers of larvae at the end of the experiment. Subklew (1934*a*) carried out similar experiments using soils of different water holding capacity in the various sections of the apparatus. His results, although only based on small numbers, seemed to indicate that the largest aggregations of larvae occurred in the soils of highest water-holding capacity.

The most comprehensive experiments were conducted by Campbell (1937) who used a moisture gradient of six sections. Different amounts of water were added to dry earth to produce the desired moisture percentages by weight of 4, 8, 12, 16, 20 and 24%. When wireworms were placed in the top section of 4% and the other sections were arranged in the above order, there was a rapid downward migration of the larvae into the moister sections. This downward movement was much more rapid than in the controls with a uniform moisture of 12%. When the gradient was placed horizontally, rather fewer wireworms moved out of the dry sections and this led Campbell to conclude that gravity had some effect on the migration out of dry soils. He also carried out a series of experiments in which the 4% section was placed in the centre; wireworms in this section migrated out in both directions into the moister parts of the gradient, but the larger aggregations occurred in the lower sections. It might appear superficially, therefore, that there is a normal tendency for wireworms to migrate downwards out of dry soil. However, the horizontal series also showed the same skewness of distribution within the symmetrical gradient and Campbell was careful to point out that the larger aggregations of larvae were found in those sections which were underneath when the tube was being loaded.

The latter effect, which was probably due to the vertical loading of the tubes, cannot very well be avoided if a tubular type of apparatus is used, but is eliminated in the present experiments by the use of troughs which are loaded horizontally. An experiment, designed to test the gravity responses of wireworms, from which misleading conclusions have been drawn, is described by Subklew (1934*b*). In this, a number of larvae were introduced into a cylinder half-filled with soil, and the filling of the tube was afterwards completed. Subklew noted that after several hours most of the wireworms had aggregated at the base of the cylinder, an observation which led him to the conclusion that the larvae were positively geotactic. In point of fact the downward migration was probably merely due to the fact that the downward movement had been initiated while the larvae were exposed on the surface of the soil.

In the present work the observations that wireworms migrate rapidly out of dry, and aggregate in wet soils, have been confirmed. The mechanism of this response is simple, for it has been shown that all the results can be readily explained by the differential effect of moisture on the rate of burrowing. Since the behaviour of *Agriotes* is otherwise unaffected by steep gradients of moisture, it cannot be said that there is any true 'avoidance' of dry soil. Further, all experiments involving the use of vertical and horizontal chambers pointed to the conclusion that, apart from the influence of moisture on the rate of movement, all distributions could be attributed to the random movements of the larvae; it is unlikely that larvae within the soil respond to gravity.

No attempt has been made to localize the moisture 'receptors', but since contact with water over any large area of the integument may result in akinesis, it seems unlikely that any specialized organs exist. In hypotonic solutions wireworms are constantly imbibing through the cuticle water which is subsequently excreted (Evans, 1943). Possibly then, the internal state arising from contact with water is in some respects the converse of that which develops during desiccation; and whereas water loss by evaporation is usually associated with the intense activity of the larva (Lees, 1943), this 'water-logged' condition may lead to comparative inactivity.

As regards the feeding responses of wireworms, it has been shown in mass experiments that there is a very pronounced tendency for wireworms to feed when the soil is relatively dry, the reverse being true of wireworms in wet soil. Experiments on single larvae demonstrated that this effect is in part due to the influence of moisture on the burrowing activity since thereby the probability of the larvae finding the food is increased or diminished.

Langenbuch (1932) has also examined this matter from an experimental standpoint. In his feeding experiments two sets of tubes were used; each contained a certain number of wireworms and a potato slice. In one series sand of varying moisture content was used as the medium; in the other, soil rich in humus. In the sand series Langenbuch claimed that the amount of feeding was about the same at all moistures, while in the soil series feeding activity was more conspicuous at low than at high moistures. Since the sand contained no humus, he concluded that in moist earth the wireworms were feeding on organic matter.

It has been shown here that diminished feeding at high moistures is also a characteristic effect when the medium used is sand. A close inspection of Langenbuch's photographs reveals that in his sand series feeding was in fact more intense at the lower saturations and was not as uniform as he claimed. *Agriotes* may feed on humus (although the recent results of Evans & Gough (1942) suggest otherwise), but these experiments do not prove it.

Finally, it is of some interest to compare the present results with field observations on the behaviour of wireworms. Several authors have noted that during periods of drought there is usually a tendency for the wireworm population to migrate downwards into moister soil. This behaviour, which may represent a reaction to a variety of unfavourable stimuli (e.g. humidity, moisture and possibly other factors) is often assumed to be due to the lack of soil moisture. In addition, the literature contains numerous references to the relation between soil moisture and wireworm damage to crops.

Printz & Bobinskaya (1936) found that wireworms aggregated in soils more than 50% saturated with water and observed that conspicuous damage to green crops only took place when saturation was less than 25%. These authors suggest that bare patches in cereal fields may be as much due to the dryness of the soil as to the abundance of the wireworm population. The downward migration of *A. mancus* during the drying out of the soil has been described by Hawkins (1936). Miwa & Yanagihara (1929) found that injurious elaterid larvae in Formosa caused most damage in poorly irrigated soils, and observed that the downward movement of the larvae might be as great as 17 in. during the dry season. Instances such as these could probably be multiplied. It will be clear, however, that the results presented in this account are in good agreement with these ecological observations.

SUMMARY

1. Simple methods are described by which wireworms could be offered the choice of two alternative moistures.

2. An account is given of the construction of a burrow. The extension of the burrow system by a single larva falls off with time; it is probable that under natural conditions such a system is semipermanent.

3. Wireworms migrate rapidly out of dry sand and aggregate in wet sand. This is due solely to the differential effect of moisture on the burrowing activity (ortho-kinesis).

4. Burrowing wireworms do not respond to gravity.

5. The feeding activity of a small population of larvae was found to be much greater at low than at high moistures. This is partly the result of the inactivity of the larvae when exposed to high moistures, and their consequent failure to reach the food. The presence of excess moisture, however, also has the effect of inhibiting all muscular activity, and this influences the manipulation of the mouth parts during feeding and locomotion alike.

6. These results are in good agreement with a number of purely ecological observations on wireworm behaviour.

Part of this work was carried out during the tenure of a grant from the Department of Scientific and Industrial Research.

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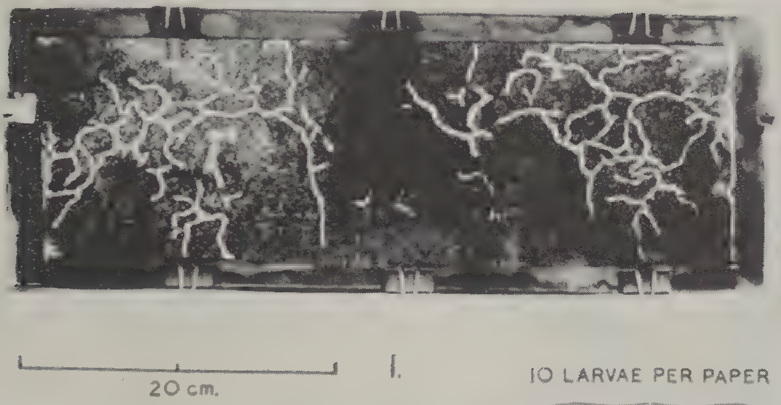
EXPLANATION OF PLATE 1

Fig. 1. The plate apparatus, photographed by transmitted light, showing wireworms burrowing in sand.

Fig. 2. A series of potato slices illustrating the effect of moisture on the feeding activity of small populations of wireworms.

Fig. 3. Feeding papers saturated with carrot juice or water showing the same effect. The papers are black-surfaced and the wireworm bites have exposed the white paper beneath.



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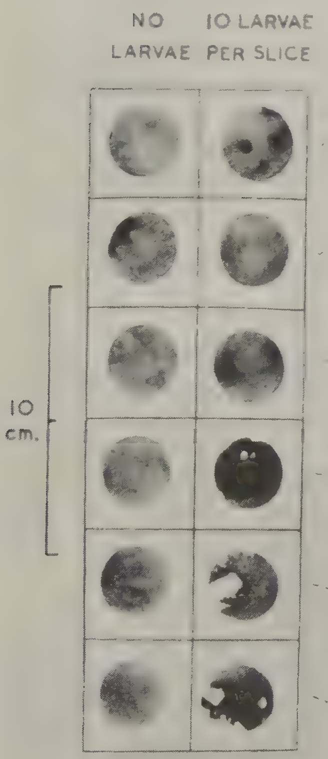

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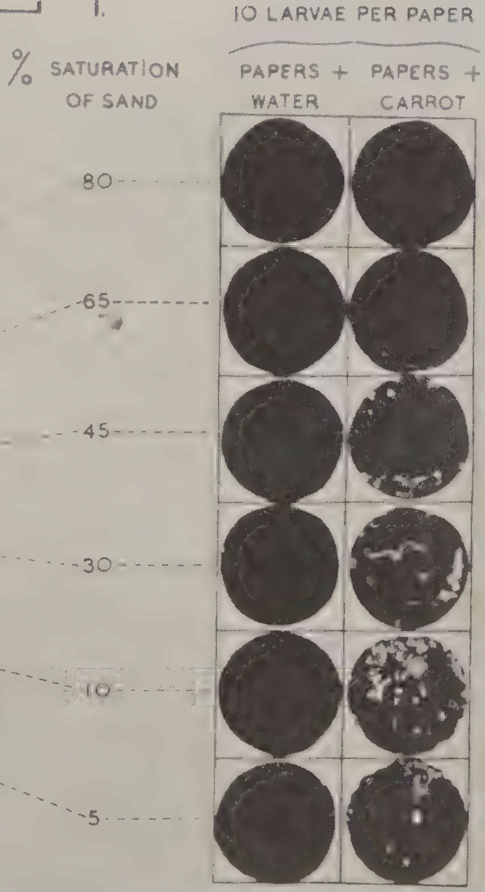


3.

EES—ON THE BEHAVIOUR OF WIREWORMS OF THE GENUS *AGRIOTES* ESCH. (pp. 43–53)

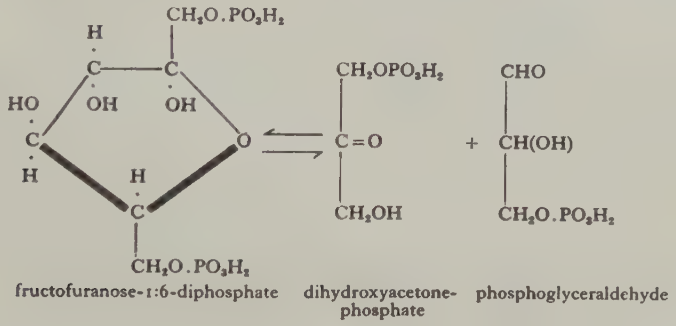
SOME EXPERIMENTS ON THE MICROSCOPICAL DEMONSTRATION OF ZYMOHEXASE IN ANIMAL TISSUES

By R. J. L. ALLEN and G. H. BOURNE,* From the Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research, Cambridge and the University Laboratory of Physiology, Oxford

(*Received 27 June* 1943)

(With Plate 2)

The presence in extracts of animal tissues and yeast of an enzyme catalysing the cleavage of hexosediphosphate (fructofuranose-1:6-diphosphate) into two molecules of triosephosphate was demonstrated by Meyerhof & Lohmann (1934*a*):



The reaction, catalysed by zymohexase, as the enzyme was called, is now generally regarded as occupying a central position in the complex series of changes by which carbohydrate is broken down in living tissues.

Meyerhof & Lohmann (1934*b*) found zymohexase to be present in extracts of various tissues of the rabbit, frog, goose and mouse, but the concentration of the enzyme was much greater in skeletal muscle of the rabbit than in any other tissue examined.

Although it is probable that in the living cell oxidation of carbohydrate can proceed in more than one way, it seems certain that the oxidation of triosephosphate and of pyruvate derived from it is a reaction of great importance in respiration. Therefore zymohexase, which is essential for the formation of these substances from hexose, must be regarded as of fundamental importance in cellular respiration.

Cytologists have long endeavoured to localize respiratory activities in certain parts of the cell. Many workers, the first being Kingsbury (1912) and the most recent Joyet-Lavergne (1938), have regarded mitochondria as centres of cellular respiration, but the evidence adduced to support this conception is very slight. In the present work attempts have been made to demonstrate zymohexase microscopically in the cells of various animal tissues, in the hope of throwing some light on the question of localization of respiration.

METHOD

The technique was based on the following points:

(1) Zymohexase is remarkably active in alkaline solution; even at *p*H 11 it retains about 50% of its activity at its optimal *p*H of about 9 (Herbert, Gordon, Subrahmanyan & Green, 1940).

(2) Further enzymic breakdown (oxidation) of triosephosphate formed under the influence of zymohexase can be prevented by the presence of iodoacetate.

(3) Inorganic phosphate is rapidly liberated from triosephosphate in alkaline solution at room temperature, whereas hexosediphosphate is stable under these conditions (Meyerhof & Lohmann, 1934*a*, *b*).

(4) Except in the presence of a large excess of inorganic phosphate hexosediphosphate and triosephosphate are not precipitated from aqueous solution by magnesia mixture (magnesium chloride and ammonium hydroxide), whereas inorganic phos-

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phate is rapidly thrown down as magnesium ammonium phosphate.

(5) Magnesium ammonium phosphate and cobaltous chloride form an insoluble complex, the cobalt in which can be converted to brownish black cobaltous sulphide, which is visible under the microscope, by treatment with ammonium sulphide after the method of Gomori (1941).

(6) Although Herbert *et al.* (1940) found that preparations of zymohexase from rabbit muscle were inactivated by contact with organic solvents, preliminary experiments indicated that intact tissues can be fixed in alcohol or acetone without serious loss of their zymohexase activity.

Sections of fresh frog and rat tissue and of alcohol- and acetone-fixed tissues of the rat and guinea-pig were incubated at 37° in one of a number of substrate mixtures (see below) for various periods. They were then removed, washed in many changes of diluted magnesia mixture (5 ml. mixture to 100 ml. water) for varying times (on one occasion for 18 hr.) to remove any hexosediphosphate absorbed by the tissues, placed in a 2% solution of cobaltous chloride for 5 min., washed in at least three changes of distilled water for a total time of 15 min., placed in ammonium sulphide solution for 5 min., washed in distilled water, dehydrated, cleared and mounted in balsam.

It was expected that the following sequence of changes would take place in the tissues during incubation: (1) formation of triosephosphate from hexosediphosphate in the presence of zymohexase, (2) liberation of inorganic phosphate from triosephosphate in the presence of OH' ions (from the ammonia in the magnesia mixture), (3) precipitation of inorganic phosphate as magnesium ammonium phosphate.

Reagents used

Sodium hexosediphosphate, 4% solution. This was prepared by treating calcium hexosediphosphate with sodium oxalate.

Magnesia mixture (Kursanov's (1938) *formula).* 5·5 g. $MgCl_2.6H_2O$ and 7 g. NH_4Cl were dissolved in 35 ml. $5N$ NH_4OH, let stand 1 hr., and filtered. 60 ml. of $4N$ NH_4OH were then added.

Purified sodium hexosediphosphate solution. Formula I: 40 ml. 4% sodium hexosediphosphate solution and 20 ml. magnesia mixture were mixed, let stand 30 min. and the precipitated inorganic phosphate present as impurity filtered off. *Formula II:* 20 ml. 4% sodium hexosediphosphate, 20 ml. distilled water and 20 ml. magnesia mixture were mixed and treated as for formula I.

Sodium iodoacetate, 0·1 M solution. 1·86 g. of iodoacetic acid were neutralized to bromothymol blue with N NaOH and diluted to 100 ml.

Sodium fluoride, 0·1 M solution.

Cobaltous chloride, $CoCl_2.6H_2O$, 2% *solution.*

Ammonium sulphide solution. Freshly prepared by diluting 1 ml. of the concentrated light yellow solution (prepared as directed by Treadwell & Hall, 1930) with about 50 ml. distilled water.

The substrate mixture

Three substrate mixtures were used:

A. 10 ml. purified sodium hexosediphosphate (formula I), 5 ml. H_2O, 1·7 ml. sodium iodoacetate.

B. 10 ml. purified sodium hexosediphosphate (formula I), 3·3 ml. water, 1·7 ml. sodium fluoride, 1·7 ml. sodium iodoacetate.

C. 15 ml. purified sodium hexosediphosphate (formula II), 7·5 ml. water, 2·5 ml. sodium iodoacetate.

Sodium fluoride was added to B to prevent hydrolysis of hexosediphosphate under the influence of phosphatase. It was subsequently found unnecessary since under the conditions used the tissues showed no phosphatase activity.

RESULTS

Experiment 1

Skeletal muscle, spinal cord and trachea of a 3 weeks' old rat were fixed for 24 hr. in 80% alcohol, passed through absolute alcohol to xylene (12 hr.) and embedded in wax at 56° C. Sections (10 μ) were floated on to the slides with warm water, dried at 37° C. for 24 hr. and stored at 3° C. for 3 weeks. Before use they were passed through xylene and alcohol to water. The sections of muscle were incubated for 30 min. in substrate mixture A, the sections of spinal cord and trachea for the same time in substrate mixture B.

Following treatment with cobaltous chloride and ammonium sulphide the muscle was found to be very little darker than the controls (see below). With the trachea there appeared to be an aggregation of dark particulate matter especially located round the cartilage cells; this reaction was probably spurious. The spinal cord gave practically no reaction.

It was thought that the negative reaction with these tissues was probably due to inactivation of zymohexase during the pre-treatment, which included embedding in wax. In the subsequent experiments the sections were not embedded but cut on the freezing microtome.

Experiment 2

Heart muscle, skeletal muscle, small intestine, brain, liver, kidney, skin, lung, rectum, salivary gland and stomach of a week old rat were fixed in 80% alcohol for 24 hr. at room temperature, washed in water for 5–10 min., frozen and sectioned, and the sections dropped into substrate mixture A. Sections were removed and examined after 1 and 2 hr.

Heart muscle. 1 hr.: browned diffusely. 2 hr.: slight browning in most sections, stronger browning in patches.

Skeletal muscle. 1 hr.: as heart muscle. Even under oil immersion individual fibres showed a homogeneous brown appearance; there appeared to be no partition of brown material between fibrillae and sarcoplasm (Pl. 2, fig. 2).

Small intestine. 1 hr.: an irregular dark precipitate formed over the surface of the section, which was itself browned diffusely. 2 hr.: precipitate formed over most of section, strong browning of muscular coat, epithelium also darkened.

Brain. 1 hr.: very faint browning. Cerebellum and cerebrum stained with the same intensity. In he cerebellum the Purkinje cells were darker than other elements. In general all the cells seemed to be lightly darker than the surrounding fibres. Under oil immersion the cytoplasm of the cerebellar cells ppeared to contain large dark circular bodies of which there were more than one to a cell. 2 hr.: more intense reaction, distributed as before.

Liver. 1 hr.: light browning. The surface of the section was covered with dark precipitate. 2 hr.: same as at 1 hr.

Kidney. 2 hr.: some dark precipitate, some browning, which was more intense in some glomeruli han in the tubules.

Skin. 1 hr.: slight browning. Cells of hair ollicles slightly darker than other parts of the section. 2 hr.: same as 1 hr.

Lung. 1 hr.: no reaction. 2 hr.: no reaction.

Rectum. 1 hr.: light browning of smooth muscle coat and slight browning of submucous connective issues. 2 hr.: same as at 1 hr. except that slight rowning of some deeper epithelial cells.

Salivary gland. 1 hr.: no reaction.

Stomach. 1 hr.: light browning of smooth muscle coat and submucous connective tissue near the muscle; no reaction in epithelium. 2 hr.: same as at 1 hr.

It is believed that the browning observed in the arious tissues in this experiment was due to the ctivity of zymohexase.

Experiment 3

In this experiment substrate mixture C, containing lower concentration of hexosediphosphate than A r B, was used. The extraneous dark precipitate ormed over the surface of some of the sections in xp. 2 was absent.

Sections of some of the tissues of the same animal sed for Exp. 2 were used. The procedure was as n Exp. 2 but in some cases (indicated below) washing after incubation and before cobalt treatment was continued for much longer than usual.

Kidney. 1 hr.: general light browning, intense immediately beneath capsule. Some small areas of he section showed very dark staining.

Intestine. 1 hr.: slight browning; smooth muscle oat darker than mucosa (Pl. 2, fig. 1).

Liver. 1 hr.: moderate browning. There was a radation in the intensity of staining, the reaction being faintest with the peripheral cells of the lobules and increasing progressively in intensity as the central veins of the lobules were approached (see Pl. 2, fig. 3). 3 hr.: washed overnight before cobalt treatment. Moderate browning. With this section large numbers of lilac-coloured crystals were observed on the surface of the tissue after cobalt treatment but before sulphide treatment. The nature and cause of this curious artefact is unknown.

Skeletal muscle. 2 hr.: very dark diffuse browning. 3 hr.: washed overnight before cobalt treatment. Same as at 2 hr.

Brain. 2 hr.: moderate browning of both fibres and cells. 3 hr.: washed overnight before cobalt treatment. Browner than at 2 hr.

Experiment 4

Pieces of liver, brain, skeletal muscle and adrenal from a 3 weeks old rat were treated as in Exp. 3 except that they were fixed in absolute acetone instead of alcohol. They were incubated for various times in substrate mixture C.

Liver. 1 hr.: slight browning and slight extraneous dark precipitate. 4 hr.: much darker than at 1 hr.

Brain. 1 hr.: slight browning and slight extraneous black precipitate. 4 hr.: much darker than at 1 hr.

Skeletal muscle. 1 hr.: moderate browning.

Adrenal. 4 hr.: slight browning. Slight extraneous dark precipitate.

Controls

Controls of two types were used. First, sections of all the specimens used were passed through the cobalt chloride and ammonium sulphide reagents in order to demonstrate any preformed inorganic phosphate in the tissues (Gomori, 1941). In all cases no reaction was obtained. Secondly, sections of all the specimens used were treated by Gomori's (1941) method for demonstrating alkaline phosphatase. The appearance of these sections (see Bourne, 1943) was so different from the zymohexase preparations as to exclude the possibility of the present results being due to alkaline phosphatase activity.

DISCUSSION

The strong browning of skeletal muscle with the present technique is in accordance with the well-known fact that this tissue is especially rich in zymohexase. This also affords important additional evidence that the reactions obtained were not due to alkaline phosphatase, because skeletal muscle contains very little of this enzyme as compared with, for example, kidney. The positive results with heart muscle were also to be expected.

Transverse sections of the skeletal muscle preparations when examined under an oil immersion objective showed no difference in intensity of reaction between the fibrils and the sarcoplasm; this is perhaps surprising in view of the fact that zymohexase is usually considered to be associated with the more soluble parts of the muscle tissue. This result may, however, be due to the limitations of microscopical resolution, or to diffusion of some of the reagents.

In all the preparations from the alimentary tract the smooth muscle coat reacted more intensely than any other part of the section. This suggests that smooth muscle contains appreciable amounts of zymohexase, an observation which does not appear to have been previously recorded.

In the cells of all organs which gave a positive reaction the brown colour was diffused throughout the cell and did not appear to be localized in any particular part of the cell. Certain cells in the brain were exceptional in containing darker staining bodies in the cytoplasm, but even here the rest of the cells stained a dark brown as well. These results suggest (assuming they are not due to diffusion of the reagents and we believe they are not) that the respiratory activity of the cells, at least as regards the metabolism of carbohydrate, is not a property of any particular cell element but is a property of both the nuclei and cytoplasm as a whole.

The presence of an extraneous dark precipitate covering the surface of some sections after incubation with the more concentrated substrate mixtures, and subsequent treatment, is difficult to explain. It occurred independently of whether the sections showed browning or not and, therefore, its occurrence does not invalidate the results obtained, but its presence indicates that the technique requires further investigation before it can be recommended for general use.

SUMMARY

A technique is described for the microscopical demonstration of zymohexase (an important enzyme in the breakdown of carbohydrate in living tissues).

The technique involves incubating sections of tissue in a substrate mixture containing hexosediphosphate in the presence of magnesia mixture and iodoacetate, followed by treatment of the magnesium ammonium phosphate so formed with cobaltous chloride and ammonium sulphide.

The enzyme was found by this method to be present in skeletal, heart and smooth muscle. In cellular organs it was found to be diffused more or less uniformly throughout the cell.

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EXPLANATION OF PLATE 2

Fig. 1. Section of jejunum of rat, showing dark staining of the smooth muscle layer and no reaction in the villi. × 80.

Fig. 2. Skeletal muscle of rat. Showing uniform dark coloration of the fibres. × 800.

Fig. 3. Liver of rat. Showing diffuse darkening of cells around the central vein. The particulate black material in the section is extraneous precipitate deposited on top of the section. × 40.

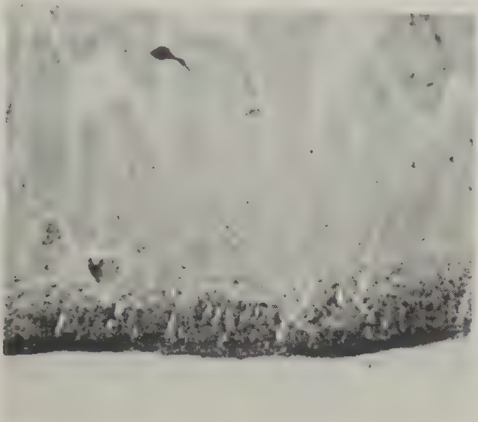

Fig. 1

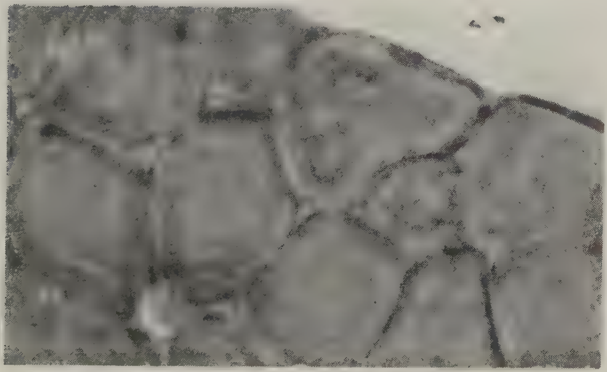

Fig. 2

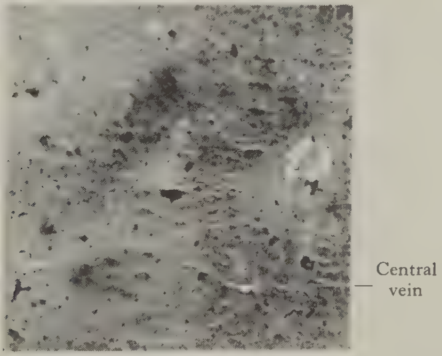

Fig. 3

LLEN AND BOURNE—SOME EXPERIMENTS ON THE MICROSCOPICAL DEMONSTRATION OF ZYMOHEXASE IN ANIMAL TISSUES (pp. 61–64)

THE BEHAVIOUR OF A POPULATION OF HONEYBEES ON AN ARTIFICIAL AND ON A NATURAL CROP

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(*Received* 4 *July* 1943)

(With Six Text-figures)

INTRODUCTION

The behaviour of a population of honeybees on a crop is a problem of considerable theoretical interest and practical importance, and one which has so far received little attention. Experimental work has revealed much about the behaviour of the individual honeybee in respect of the method of communication between individuals, colour vision, olfactory and gustatory perception, etc. (Von Frisch, 1914, 1919, 1921, 1923, 1930), time sense (Kalmus, 1934), water perception sense (Hertz, 1934), etc., without, generally speaking, dealing with the problems of the population as a whole. However, Minderhoud (1931) published field observations on the area ranged over by a foraging bee working dandelion, clover and various cultivated plants, and reached the important conclusion that bees return time after time, even day after day, to an area of the crop a few yards square, probably not more than 10 yd. each way. Similar observations made by Buzzard (1936) using marked bees went even further than those of Minderhoud, and showed that the same bees return continually to the individual plant upon which they were first observed. Each plant appeared to have its own population of bees which only trespassed on to a neighbouring bush of the same species when the branches of the two bushes actually interlaced.

Counts of bees on crops have, of course, been made, but their interpretation has usually proved difficult in the light of past knowledge and has seldom been correlated with experimental work upon the behaviour of the individuals of which the population was composed. These difficulties of interpretation have probably been largely due to the considerable differences existing between the conditions under which behaviour experiments are usually carried out and the complex conditions appertaining to the field. Experiments under conditions that are more nearly 'natural' than those under which observations have hitherto been made, but with conditions still under a considerable degree of control, may be expected to lead to a better understanding of the behaviour of the populations as a whole. This was achieved by using artificial nectar sources (Petri dishes) distributed over a large field. The obvious advantages of the use of these artificial nectar sources were that the behaviour of a small number of bees to an individual dish is already well known, that the concentration, quantity and time of presentation of nectar (sugar syrup) can be varied at will, and that the density of distribution of the nectar sources in the field, and their position relative to the colonies of bees, can be arranged according to the purpose of each experiment.

If the differences as appreciated by the bees between dishes and flowers were fully understood, certain conclusions concerning the behaviour of bees on natural crops could be drawn with confidence; however, as this is not so, experiments on the behaviour of a population on natural crops had also to be undertaken before any definite conclusions could be drawn.

MATERIAL, METHODS, ETC.

Experiments were carried out during the summer of 1942 in a large field on the farm of the Rothamsted Experimental Station, next to the Home Apiary, which throughout contained about thirty-five colonies of bees of Caucasian, Italian and Caucasian-Italian strains.

The main observations were made in the field containing the artificial nectar sources and upon two natural crops. The artificial nectar sources (Fig. 1) were arranged so that they occupied part of a sector, of about 30°, of a circle the centre of which lay near the middle of the Home Apiary. The field containing the artificial nectar sources was fenced pasture, with few flowers, crossed by several footpaths and containing a few small clumps of trees. 112 Petri dishes were placed in the experimental field, arranged in thirteen curved rows on arcs of circles centred in the Home Apiary. The nearest of these curved rows was situated at a distance of 160 yd. from the centre of the apiary, and the most distant curved row was 400 yd. away. The radius of curvature of each row was increased by 20 yd. over that immediately preceding it. The dishes in each row were likewise placed 20 yd. from one another.

Each artificial nectar source consisted of the top or bottom section of a Petri dish in which a 1 lb.

honey jar full of sugar solution was inverted to act as a reservoir. A piece of blue pottery was placed upon the top of each jar and a similar piece of pottery was laid on the grass nearby. No scent was added to the syrup throughout the course of these experiments.

Most of the observations on natural crops were made on a piece of common land on which large patches of willow-herb (*Epilobium angustifolium*) were growing. For the observations made upon willow-herb a patch was selected about 50 yd. long × 40 yd. wide which had more or less well-defined boundaries. This patch was about 600 yd. east of the experimental apiary and contained gorse, brambles, wild raspberries, etc., in addition to many plants of *Epilobium*.

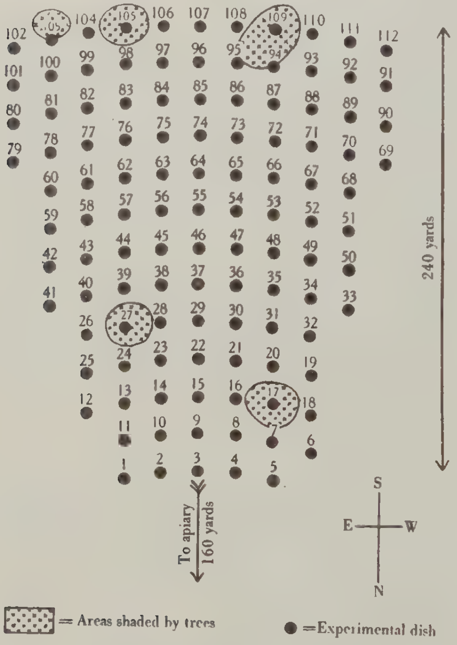

Fig. 1. Lay-out of Petri dishes on experimental field.

The second growing crop upon which observations were made was the cultivated ornamental thistle, *Echinops sphaerocephalus* L. This was growing in five isolated patches in a long border at one side of the experimental apiary, each patch being about 18 yd. from those on either side of it. Each patch was arranged to contain approximately forty flower heads at the time that the observations were made.

In order that they might readily be recognized, individual bees, whose behaviour it was desired to study, were marked with one or more brightly coloured paints, on the dorsal surface of the thorax or abdomen, or both. Since it was often desirable to mark bees in the field without catching the individuals concerned or disturbing them unduly, quick-drying paints were employed. These were prepared by mixing small quantities of dry, powdered pigments with a solution of rosin in acetone. The amount of pigment added was varied according to the intensity of colour desired, and sufficient rosin was added to produce a paint of good consistency. The bees were marked usually whilst actually on the flowers. Great care was taken to see that no paint was placed on the antennae, eyes, legs, or wings of the individuals concerned, since the presence of paint on these parts of the bee's body seriously interferes with its normal behaviour.

Marked and unmarked bees were observed, or counted, on the whole, or on a part, of one of the experimental sites as required, care being taken to interfere with their activity as little as possible. Most counts were made by one or more observers whilst walking over the field. It was seldom possible, nor indeed necessary, to station an observer at each dish.

The concentration and quantity of the sugar syrup at the artificial nectar sources was varied according to the requirements of particular experiments.

EXPERIMENTS AND RESULTS

(i) *Indications of constancy to a particular site in an artificial crop*

On 5 June 1942, a good flying day, two bees found visiting dish no. 85 on the experimental field (Fig. 1) 360 yd. from the apiary, were marked for purposes of recognition, their identification marks being *Y* and *YY*. A constant supply of sucrose solution (about 12% sugar) was maintained in this dish throughout the next few days. Between 11.26 a.m. and 12.30 p.m. (G.M.T.) continuous observation was kept on this dish and the number, duration and time of the visits of these two bees (*Y* and *YY*) to the dish were noted. The results obtained are shown in Table 1. Throughout the period of observation, bee *YY* chose a point on the west side of the experimental dish for feeding, and *Y* chose a point on the north side. Neither bee moved so much as an inch from its chosen position and each assumed a characteristic attitude while drinking, *Y* drinking from the side of the dish and *YY* settling head downwards on the reservoir.

At 4.25 p.m. (G.M.T.) *Y* and *YY* were still paying regular visits to the dish and must between them have already made at least 150 trips almost certainly without a break. At 9.20 a.m. (G.M.T.) on the following day these two bees were still visiting the dish with great regularity.

It should be pointed out that the two bees in order to reach their chosen dish 360 yd. from the apiary had to fly over or near a number of exactly similar dishes filled with syrup of the same concentration as that in the chosen dish, and were thus always in a position to choose to collect syrup from other dishes, in much the same way as in nature they would be free to collect nectar from any one or more of a number of flowers composing a crop.

At about 1.55 p.m. on the day on which observations given in Table 1 were made, the experimental dish was moved a farther 2 yd. away from the apiary at a time when both bees (*Y* and *YY*) were absent. Bee *Y* returned to the old site, flew diagonally across it and had found the dish within 12 sec. of crossing its original site, *YY* similarly crossed the original site and located the dish 7 sec. later.

Table 1. *The number, duration and time of visits of two honeybees (Y and YY) to a dish of syrup, 5 June 1942*

Identity of bee	Time of visit (G.M.T.)	Duration of visit	Interval between visits
	hr. min.	sec.	min.
YY	11 29·9 a.m.	31	—
Y	11 33·5	40	—
YY	11 34·2	30	4·3
Y	11 38·1	41	4·6
YY	11 38·6	30	4·4
Y	11 42·0	30	3·9
YY	11 43·3	30	4·7
Y	11 46·0	38	4·0
YY	11 48·8	36	5·5
Y	11 51·0	43	5·0
YY	11 53·7	30	4·9
Y	11 55·3	37	4·3
Y	11 58·3	37	3·0
YY	11 58·3	32	4·6
Y	12 1·2 p.m.	40	2·9
YY	12 2·0	30	3·7
Y	12 4·0	40	2·8
YY	12 5·2	28	3·2
Y	12 7·5	41	3·5
YY	12 9·3	35	4·1
Y	12 11·3	40	3·8
YY	12 13·0	32	3·7
Y	12 14·2	42	2·9
YY	12 17·5	35	4·5
Y	12 17·8	38	3·6
Y	12 22·0	44	4·2
YY	12 22·5	33	5·0
Y	12 26·0	46	4·0
YY	12 28·3	38	5·8
Y	12 30·0	40	4·0

Mean length of time between visits *Y* = 3·77 ± 0·17 min., *YY* = 4·49 ± 0·20 min.

Mean length of time spent drinking *Y* = 39·81 ± 0·89 sec., *YY* = 32·28 ± 0·75 sec.

The remarkable constancy of the behaviour of individual bees, such as is shown in Table 1, has been used by one of the authors (Kalmus, 1938) for investigating the internal urge which compels a nectar-collecting bee to fly either to or from the hive. However, the very definite differences between the individuals—*Y* being the quicker at flying but the slower at drinking would indicate some possibilities of further research. In addition, there are indications of similar trends in the time series of the two individual, which hint at a dependence of the visits on environmental factors.

A similar experiment using four other marked bees gave very similar results. Therefore it appears that so long as there is an abundance of nectar at a given source, bees will confine their visits to this one source for many hours and even over two consecutive days.

(ii) *The territorial constancy of a small population of bees marked near the centre of a group of dishes in the experimental field*

For several days prior to this experiment bees were fed at all the dishes in the experimental field, but care was taken to see that the syrup in dishes 84 and 85 did not become exhausted. On the evening of 25 June about one hundred bees feeding at these two dishes were marked. Fig. 2 shows the subsequent distribution of these marked bees on dishes 84 and 85 and the fifteen dishes nearest to them, as determined by eleven counts made at various times on the following day. Care was taken to see that all seventeen dishes upon which the counts were made were continuously supplied with syrup during this day.

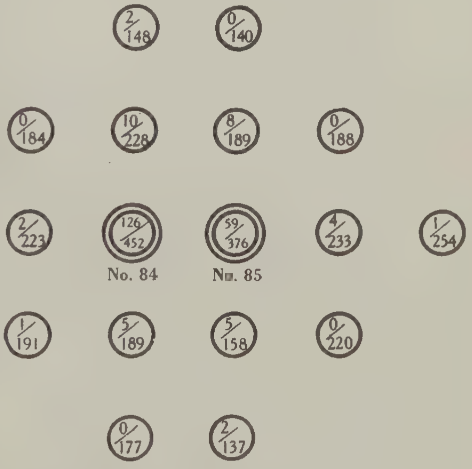

Fig. 2. Distribution of bees counted in eleven counts on seventeen dishes 26 June. The bees previously marked at dishes 84 and 85 are indicated in the numerators, total unmarked and marked in denominators.

As will be seen from Fig. 2 the marked bees exhibited a high degree of constancy in their visits to the dishes where they had been marked (nos. 84 and 85); however, some of the bees were recovered on the surrounding dishes, and of these most were found upon the dishes nearest to dishes 84 and 85 upon which they had been marked. This may be a demonstration of some degree of spreading subsequent to the marking, or alternatively these marked bees found on dishes other than 84 or 85 could be supposed to have been practically constant in their attention to the various other dishes upon which they were found, and merely to have wandered temporarily to dishes 84 and 85 at or about the time of marking. Either supposition entails the assumption of a certain small degree of inconstancy to a particular dish. This inconstancy may well have been induced by failure of the supply of syrup in certain dishes overnight (25–26 June) and neglect to refill them before the bees commenced to visit them next morning, so that bees at some dishes finding the supply at their customary site temporarily suspended had searched for and found other adjacent sources.

During the next 3 days no syrup was supplied in any of the dishes. On the following 3 days syrup was again supplied continuously in all dishes and a further eighty counts were made during this subsequent period. The results of these counts are given in Fig. 3, from which it will be seen that even after a 3-day interruption in the supply of syrup, the marked bees which were recovered were found close to the original site of markings.

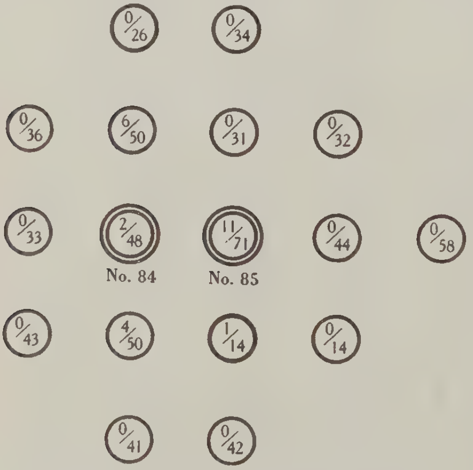

Fig. 3. Eighty counts made on the same dishes as in Fig. 2, 30 June–2 July inclusive, after three complete days without feeding.

(iii) *Visits of bees to a new source of syrup placed between previously established sources, and the effect when the established sources dry up*

Fig. 4 shows the distribution of bees on filled dishes spaced along a line at approximately 1 yd. intervals, running due south from the hives. On the previous days the dishes had been in groups over areas about 1½ yd. square at places *A*, *B* and *C*, with no dishes in between. (*Note*. The arrangement of the dishes for this particular experiment was different from that of the experimental field previously described and used for subsequent experiments.) The graph (Fig. 4—dots) which shows the results of a count made 1¼ hr. after the dishes had been spaced at the 1 yd. intervals, and filled, shows clearly that practically all the bees came back at or near to the three old sites, their numbers decreasing rapidly with distance from these sites. These dishes were all on a substantially uniform stretch of meadow, and there were no local landmarks by which, to human eyes at least, the old sites were noticeably different from the intervening spaces. During the 1¾ hr. following this count, the bees were allowed to finish up all the syrup in most of the dishes along this line, and the points marked with crosses on the graph show the numbers of bees on the various dishes half an hour after refilling all the dishes (about 2¼ hr. after the first count). These latter counts show great local variation, but the peaks at the old sites have now entirely disappeared. This can be explained by (1) the observed fact that the bees, when they had finished all the syrup at any one dish, after a time began to seek out other dishes, which necessarily proved to be some of the intermediate ones; and (2) the assumption that thereafter some of the bees retained a memory for the intermediate dishes they had found, and revisited them even after the dishes at the original sites had been refilled.

The work of Wolf (1926–31) may possibly throw some light on the fact that a bee is able to locate a dish at a given distance from its hive even when that dish is placed on a uniform piece of land which bears no distinctive marks by means of which the bee could memorize the site. Wolf, in his work on the home-finding sense of the honeybee, demonstrated that a bee possesses a definite sense of distance as well as of direction. On leaving the hive to visit a particular source of nectar she will fly in a more or less straight line for a definite distance in a given direction, and not until she is within a few feet of a position the correct distance from the hive will she deviate from a straight line and commence to make searching flights.

In order to explain more fully the behaviour of a bee when the food supply in a dish which it had grown accustomed to visit failed, let us assume that counts made at a given dish showed an average of twenty bees feeding there at any time during flying hours. The total number of bees visiting this dish would then be several times as great, on account of the fact that of the total time spent by a bee on any one foraging trip only a small proportion of this (according to conditions usually ½–1¼ min.) was spent in drinking and the greater part of the time in flying to and from the hive and regurgitating the syrup collected.

As soon as the syrup in a dish begins to dry up, the bees can no longer fill their honey-stomachs with the same rapidity as before and, as a consequence, have to spend a considerably longer time upon the dish in order to acquire a load. As a result of this the number of bees on the dish at any one time gradually increases as the ratio of the time required to collect a load of syrup and the time spent in flying to and from the hive is increased. Even when no syrup remains in the dish at all, the bees accustomed to feed at this dish eagerly search the site for syrup. However, after a few minutes most of them begin searching round the empty dish in concentric circles of ever-increasing size, but at intervals still returning to the dish, until they find a new dish or other source of food. Gradually the number of visitors to the dry dish decreases from this cause, and only occasional visitors persist in visiting it at longer and longer intervals, the frequency of such revisits depending upon the extent of the previous conditioning to this site, competition and other factors.

It should be pointed out that, although this behaviour is in principle probably the same as that which occurs on a natural crop of flowers when the nectar supply fails, the time relationships are not the same on a flowering crop as in the case of the dishes of syrup, since on flowers the time spent by the bees in collecting a load of nectar is usually greater than the time spent in flying to and from the hive. This ratio between time spent upon the crop and time spent flying, etc., will, of course, vary not only with the crop but also under different climatic conditions which will in their turn greatly influence the abundance and concentration of the nectar supply.

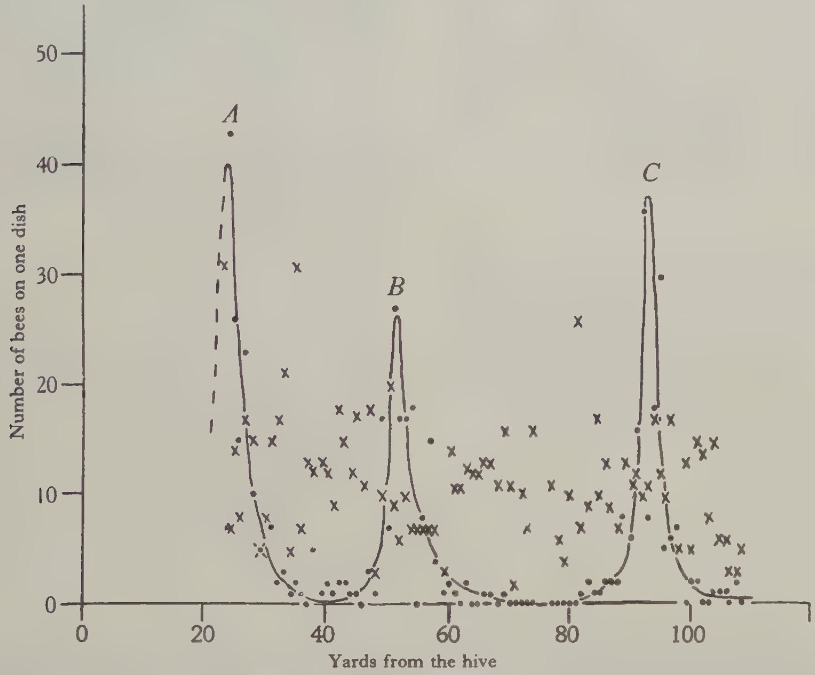

Fig. 4. Distribution of bees on a single line of Petri dishes, at the sites of some of which they had been previously fed. ● = 1½ hr. after the dishes had been spaced out along the line. × = 2¼ hr. after that, when most dishes had dried up and had been refilled again.

(iv) *Some of the influences of the situation of a dish, distance of a dish from the hive, and weather conditions, upon the distribution of a population of bees in the experimental field*

(*a*) *Number of bees visiting dishes placed in shady surroundings*

The number of bees visiting dishes placed in the shadow of trees and other foliage was found to be very much smaller than was the case with dishes in more open surroundings. Three dishes (nos. 17, 27 and 109) situated among small groups of trees in the experimental field were very rarely visited by any bees. The number of bees visiting dishes situated in partial shade (nos. 7, 94 and 105) was also small. Table 2 shows the results of forty-one counts made on all the dishes in the experimental field, which illustrate these points.

Table 2. *The effect of total and partial shade on the number of honeybees visiting dishes*

	No. of dish	No. of bees	Average no. of bees per dish for all counts
Dishes in complete shade	17	6	2·7
	27	2	
	109	0	
Dishes in partial shade	7	40	32·7
	94	37	
	105	21	
Dishes in open positions	Remaining 106 dishes in experimental field	17,157	161·9

Some observations were made which appeared to indicate that fewer bees visited the dishes placed on a line drawn from the apiary to one of the groups of trees in the experimental field, over distances within about 50 yd. of the group. Unfortunately, the data were insufficient to enable a definite conclusion to be reached. If in fact such a conclusion were reached it would indicate the reluctance of bees to fly in the direction of groups of tall trees, and their preference to direct their flight towards an open area.

(*b*) *Range of feeding and weather conditions*

It is well known that wet or stormy weather usually reduces the total number of foraging bees present in the field at any given time. A heavy cloud passing over the sun is sufficient to cause large numbers of bees to return to their hives. It has also sometimes been suggested that such unfavourable conditions also reduce the distance from the hive which foraging bees will fly, causing the whole foraging population to work that part of the crop nearest to the hive. Unfortunately, it was not possible to establish definite conclusions on this point, but some data were collected which indicate that sudden changes in the weather do not cause individual bees which have become established at the more distant feeding places to leave them in favour of others nearer home. All the evidence points to the conclusion that if a sudden deterioration in the weather causes a proportion of the foraging population to return to the hive, this returning population is mainly composed of the bees which were working farthest afield, and that having returned to the hive they do not leave it again until weather conditions improve sufficiently for them to go back to their original sites. The foraging distance of the individual bee is not apparently reduced, but the more distant foragers are the more likely to return to the hive until conditions improve.

(*c*) *Effect of tall herbage surrounding feeding places*

Some of the dishes in the experimental field were situated in the middle of nettle-beds and other rather tall herbage. Dish 95, for example, was rather hidden amongst plants growing about 2 ft. high. It was found that very few bees visited this dish and it often remained neglected, although full of syrup, even when the food supply in the other neighbouring dishes had failed. However, on 3 June several bees found this dish and thereafter it was visited quite frequently. It would appear that the earlier neglect of this and similarly situated dishes was caused not so much by the slight shade thrown by the surrounding herbage, as by the fact that this dish was somewhat hidden.

There were other local factors, such as sheep troughs, paths and fences, in the experimental field, which sometimes apparently exerted some influence on the number of bees which visited the dishes nearest to them, but little more than indications on these factors could be obtained.

(v) *Effect of nectar concentration upon number of visiting bees*

On account of frequent failures in the supply of syrup in the dishes in the experimental field during the latter part of the season, a state of affairs developed such that many bees had an interest in more than one dish. This appeared to be an excellent opportunity to test the effect of using two or more concentrations of syrup in the field simultaneously.

After several consecutive days upon which syrup of a moderately high concentration was supplied in all the dishes, the dishes were all emptied on the evening of 18 June and were not refilled until 1 p.m. (G.M.T.) on 19 June by which time the total number of bees visiting the field was small. Alternate dishes in the rows nearest to the hives and alternate groups of two and three dishes at greater distances from the hives, were then filled as quickly as possible with sugar solutions of two different concentrations (7·4 and 19·4% sugar by weight). Continuous counts were then made by three observers patrolling the field from 3.30 p.m. until 6.30 p.m., when all the dishes were emptied once again. Fig. 5 shows the results of these counts.

There is no doubt that many more bees were attracted to the dishes containing the higher concentration syrup than were attracted to the dishes containing the syrup of lower concentration. The following day all the dishes were refilled with syrup of a medium concentration (13·8% sugar by weight) and counts were made. An uneven distribution of the bees on the dishes was still observed for some hours, and in the three counts made before noon a total of 960 bees were found on the fifty-four dishes which had contained the syrup of higher concentration (19·4%) on the previous day, and 534 bees on the dishes which had contained the syrup of lower concentration (7·4%).

(vi) *The area of a crop of willow-herb visited by individual bees*

The 'fixation' of honeybees to localized artificial feeding sources as shown above supports Minderhoud's (1931) observations that individual bees restrict their feeding activities to a small area when working clover, dandelion and other crops. Buzzard (1936) also reported cases of individual bees returning at frequent intervals to one particular flower or small group of flowers. We were able to obtain data supporting these observations by studying the distribution of a population of bees visiting the flowers of willow-herb and the heads of the ornamental thistle, *Echinops*.

(*a*) *Observations on willow-herb*

The following observations provide information on the local constancy of bees working on a small area of willow-herb situated in the middle of a much greater area of this plant. On this small patch of *Epilobium* (see p. 66 for description of area) which was approximately 5 × 8 yd. in extent, being somewhat separated from the remainder of this crop, most of the bees (about 100) visiting the flowers during a period of 2 hr. were marked red. Round the central point of the area counts of marked and unmarked bees were made in annular concentric zones, each zone being 1 yd. in radial width. Fig. 6 shows that percentage of marked bees counted in each of these zones at various periods after marking.

One of the marked bees was observed near the centre of the plot in company with fourteen unmarked individuals 17 days after marking. The results, although not allowing an accurate assay of the dimensions of the 'fixation' areas of individual workers, do indicate that the majority of the bees observed restricted their activities to areas of a diameter of less than 5 yd. This can be estimated from the shape of the curves in Fig. 6 where the vertical line *A* represents the minimum distance (2½ yd.) from the centre to the boundary of the marking area. Had each bee restricted its activity to one fixed point only, the curves would show fairly horizontal portions up to the limit *A* (2½ yd.) followed by a steep and rather uniform decline to zero at *B* (4¾ yd.), the maximum distance from the centre to the boundary. From the departure of the curves, drawn from observed points, from this theoretical shape it appears that the bees did move, in fact, but only rarely more than 2–3 yd., seldom more than 5 yd. Fig. 4 also indicates that 24 hr. after marking, the bees had moved farther than they had 3–5 hr. after marking.

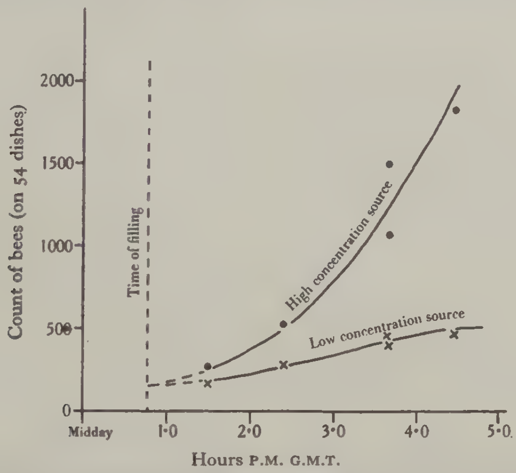

Fig. 5. Numbers of bees for a series of counts on fifty-four Petri dishes filled with 19·4 % sugar (●), and on fifty-four dishes filled with 7·4 % sugar (×), at various intervals after filling.

(*b*) *Observations on* Echinops

Observations on the local attachment of bees working isolated patches of the decorative thistle, *Echinops*, were also made. The site of these observations has been described on p. 66. The length of time spent by an individual bee on any one foraging trip on the heads of the *Echinops* plants varied between 20 and 60 min. This is very much longer than the time spent on a dish of syrup (½–1½ min.); however, the time spent in the hive depositing the *Echinops* nectar was about 3–4 min., which is of the same order as that spent in delivering a load of syrup.

By marking newcomers to three alternate plots of *Echinops* (nos. 1, 3 and 5) out of the five in the row, using different colours each day for 4 weeks, populations of marked bees built up on each plot and were remarkably constant in their attachment to their own particular plots, the individuals from which they were composed seldom visiting neighbouring plots. Bees of one marking colour were observed over periods up to 16 days, though the number of individuals of each colour present gradually decreased from day to day. After the first few days there were always more bees present which were marked with colours other than that in use of the day in question, i.e. the majority of bees present had been visiting the plot for more than 1 day.

On the two intervening plots (nos. 2 and 4) all visiting bees were caught and were not subsequently released. As a result there was always more nectar available per flower in these two plots than in the other three plots, so that bees straying to the former tended to remain there. In view of the short distance between the plots of *Echinops* and the hives, many new unmarked bees were captured there, but the proportion of bees marked at plots 1, 3 or 5 to stray over the 18 yd. distance between plots was only of the order of 10% per day. Thus the observations made upon isolated plots of *Echinops* also revealed a considerable, but not absolute, degree of constancy of visit of the individual bee to a small area, as did the experiments with dishes and the observations on the small area of willow-herb situated in the middle of a much larger area of this plant.

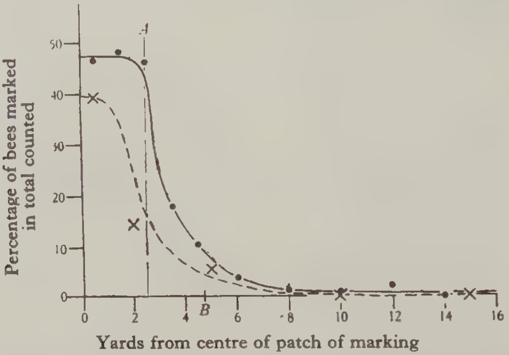

Fig. 6. Percentage of marked bees against distance from the centre of an oblong area studded with *Epilobium*, on which they had been marked previously. Each point is based on a count of at least fifty bees, usually between 100 and 200. ● = bees counted 3–5 hr. after marking; ✖ = bees 24 hr. after marking.

DISCUSSION

Most of the findings in this paper are self-explanatory, and practical deductions can be drawn from them. However, it should perhaps be stressed again that there is one important difference between experiments with artificial sources of nectar and experiments with natural sources of nectar; the short time spent by a bee on a dish does not correspond with natural conditions, where the time spent, per trip, in collecting from flowers is very much greater even under the most favourable conditions.

Lundie (1925), using an automatic bee counter, found that under the conditions prevailing at the time that his observations were made, the bees spent between 8 and 104 min. on any one foraging trip, according to conditions. This is in approximate agreement with the 20–60 min. periods which were our usual findings on *Echinops*, and is many times longer than the time spent on the syrup dishes.

As a consequence of the difference in the time spent by the bees on an artificial as compared with a natural source of nectar, it could be shown that only a small proportion, differing with conditions, but approximately a seventh, of the bees collecting syrup from a particular dish were present at any one time. The proportion of the fixed population present upon a given patch of flowers in good weather was always much larger, on account of the very much greater time the individual bee spends in collecting a load. This agrees with the observation that no corresponding increase in time is taken in discharging the load. However, it is difficult to estimate with any accuracy the relation between the time spent by the bees in the hive and on the crop on any given day, nor do Lundie's or other observations give any certain clue. So long as this is not known, no statements can be made as to the number of bees permanently attached to a given area, nor can any variation in local constancy of individual bees be determined.

The fact that foraging honeybees work only a limited area has an important bearing on the genetics of those flowering plants which they pollinate. If such a species is self-sterile and covers a large coherent area in great density it will still not form a homogeneous interbreeding population but rather an aggregate of small visiting systems in the sense of Wright (1921) which do not exceed a few yards in diameter. In short the local constancy of bees results in a considerable degree of inbreeding in the pollinated plants.

SUMMARY

One hundred and twelve Petri dishes filled with sugar syrup were arranged at 20 yd. intervals from each other in a meadow. Individual bees were observed to visit one chosen dish with great regularity for one or more days, provided that the supply did not become exhausted.

Occasionally bees marked on one dish were observed to visit an adjacent dish. This occurred most frequently when the supply of syrup temporarily failed at the original site of feeding, but, even after a 3-day interruption in the syrup supply bees often returned to feed at the original site.

Bees feeding at a dish full of syrup spent only a fraction of their time (about 1 min.) per visit drinking, but several times as long flying to and from the hive and delivering their load.

When the supply of syrup in a dish became exhausted all the bees accustomed to visit that dish gradually accumulated there impatiently seeking for food; after some minutes they extended their radius of search, and many located another source near at hand. Thereafter they visited either the new source or the old, or both, when the syrup at the original site was replenished. The nearer such a new source was to the original one the more likely a bee was to find it; a dish 20 yd. away from the original site was quickly found despite the fact that such a second dish would seldom be visited if the syrup at the original site was constantly maintained.

Bees were deterred from collecting syrup from dishes placed even partially in shade; they very seldom worked beneath the shade of trees. There were even some indications that they prefered not to fly in the direction of shady trees.

Over the range of distances covered (160–400 yd.) there were always more visitors to the nearer than to the more distant dishes. The extent of this difference, however, varied from day to day. Bees accustomed to collect syrup from the dishes farthest from the hive did not move to sites nearer home when the weather became unfavourable. There was some evidence, however, that bees working a long way away from the hive were more easily deterred from foraging by unfavourable weather than those working close to the apiary.

When two different concentrations of syrup were offered in different groups of dishes simultaneously the number of visitors to the dishes containing the syrup of high concentration rose considerably higher than that of the visitors to the dishes containing low-concentration syrup; even after all the dishes had been refilled with syrup of uniform concentration on the following day, this difference remained noticeable.

Bees marked on a patch of willow-herb (*Epilobium angustifolium*) situated in the midst of a large crop of this plant, were usually recovered within 5 yd. of the point of marking. Such bees remained 'fixed' to this area for several days. Observations were made upon isolated patches of the cultivated thistle, *Echinops sphaerocephalus*, of bees which continued to visit the patches upon which they were marked for periods up to 16 days. The majority of the bees working the patches showed great constancy: and of such regular visitors the percentage per day observed to stray to other patches of *Echinops* 18 yd. away was comparatively small.

The time spent by foraging bees upon the flower-heads of *Echinops* on any one visit greatly exceeded the time spent in collecting syrup from a dish (20–60 min. as against 1 min.); but the time spent in flying between the hive and the flowers, or dish, and unloading was approximately equal in each case. Only a small proportion of the population of bees working on a particular dish could be found feeding there at any one time, whereas on a patch of flowering plants, under good weather conditions, most of the population visiting that patch would be found there at any one time. This great difference in behaviour on dishes as compared with plants must be borne in mind in any attempt to draw conclusions from dish experiments as to the behaviour of bees.

We wish to take this opportunity of thanking Mr R. Green and Miss N. Watts for their co-operation in this work during their vacations, and Dr C. B. Williams for his interest and encouragement.

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MITOTIC ACTIVITY IN THE FEMALE MOUSE PITUITARY

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(With Plate 3 and One Graph)

INTRODUCTION

The stimulation of mitotic activity in the anterior pituitary has been shown in the rat (Wolfe, 1935; Halpern & D'Amour, 1936) and the monkey (Dawson, 1942). Further, a differential increase in basophil mitoses after castration (Pomerat, 1941), a variation in those of acidophils and chromophobes during the oestrous cycle (Hunt, 1942), as well as a reduction of general mitotic rate with age and a variation with the time of day (Hunt, 1943) have been observed in the rat. This work is concerned with mitotic activity in all parts of the female mouse pituitary during the normal oestrous cycle and that produced by oestrone injections during dioestrus.

MATERIALS AND METHOD

The mice selected were all about six months old and had regular oestrous cycles, of which the eight periods were determined by vaginal smears treated with haematoxylin and Pasini's stain (Bullough, 1943). Groups of five mice were injected with 0·1 mg. of colchicine in 0·25 c.c. of water per mouse at the beginning of each period and killed 9½ hr. later. As a result practically all the pituitary mitoses were in metaphase. For the oestrone series similar groups of five mice were injected subcutaneously with one to six injections of 0·25 c.c. (250 i.u.) of Menformon per mouse at intervals of 12 hr. and killed 12 hr. after the last injection, with colchicine given 9½ hr. before killing as before. The oestrone injections commenced at the beginning of dioestrus and were timed so that all mice could be killed at the same time of day (10 p.m.).

Pituitaries are not easily removed attached to the brain but if the latter be bent forward the stalk (Pl. 3, fig. 1) breaks and the whole gland can be easily detached from its membranes and lifted out, as there is no sella turcica. The tuberal lobe, which is almost devoid of mitoses in any case, is broken but can be sectioned in two halves, one attached to the distal lobe and the other to the base of the brain. The glands were fixed in Bouin and sectioned serially at 5 μ. Granulation is well shown by Anderson's Mallory but not mitoses. Haemalum and eosin is the most convenient stain for acidophil mitoses but to differentiate those of basophils from chromophobes the following method is preferable: iron haematoxylin (well differentiated), phosphomolybdic acid (4% aq. solution for ½ hr.), without washing to Mallory's blue component (½ hr. in 56° C. oven).

The number of mitoses in these pituitaries is small and with the varying dispositions of the cell types the selection of arbitrary areas for counts was impracticable. Instead each tenth section throughout each gland was examined under oil immersion and the total number of mitoses noted (see Table 1). Mitotic activity is frequently accompanied by pycnotic nuclei so that only mitotic figures in which the chromosomes could be distinguished were considered genuine.

GENERAL OBSERVATIONS

The disposition of the pituitary in the skull and the arrangement of its parts in sagittal section is shown in Pl. 3, fig. 1. The solid flattened stalk passes into a central nervous lobe, this is largely enveloped by the proximal (intermediate) lobe, which is separated centrally from the distal (anterior) lobe by the hypophysial cavity. The distal lobe enlarges greatly to either side and also extends round the edges of the cavity to meet the proximal lobe, so that the gland is considerably broader than long and is mainly composed of distal lobe. From the front edge of the distal lobe the tuberal lobe extends forwards and round the hypophysial stalk.

The nervous lobe is fibrous and well vascularized, with abundant Herring material—mostly as small blue and brown particles—which does not extend into the stalk. Nuclei are numerous, some vesicular and some rather dense, but only two mitoses have been seen throughout, one towards the centre of the lobe in the first period of dioestrus and the other (Pl. 3, fig. 9) at the periphery in the third period of oestrus. Both mitotic figures are more compact than those of proximal lobe cells and there seems no reason to suppose that they are immigrants. The internal blood supply of the proximal lobe is poor, and its cells are large and ill-defined. Some nuclei are dense and others vesicular, and they may frequently be misshapen, but in division the chromosomes are large and distinct (Pl. 3, fig. 7).

In the distal lobe the acidophils are distinct in outline, rounded to oval, with the nucleus usually to one side, and show a wide range of staining reaction depending on the number of the rather small granules they contain. When their granules are fewest acidophils approach closely to chromophobes. Their mitoses (Pl. 3, fig. 2) are quite compact but remain distinct from the pycnotic nuclei (Pl. 3, fig. 8) which often accompany them. The latter are usually solid but may break up into irregular lumps disposed peripherally in the cell. There are two unusual features in the nuclei of acidophils, one is a wrinkling and darkening of the nucleus and the other a gradual loss of staining power so that cells may have no visible nucleus at all. One or both may be degenerative but it has been impossible to determine when an acidophil is actually disintegrating. Binucleate specimens are common (Pl. 3, fig. 8), either with the nuclei pressed close together or clearly rounded off. Such may represent nuclear without cytoplasmic division but they are not noticeably more numerous after periods of mitotic activity. In distribution acidophils tend to avoid the periphery of the lobe and show their greatest concentration in the lateral enlargements. Chromophobes are granule-free cells of various sizes, usually smaller than acidophils and about twice as numerous, with vesicular nuclei, lightly staining cytoplasm and no characteristic shape. Two other cell types included here among the chromophobes are oval cells with scanty brown inclusions, possibly either acidophil granules or mitochondria, and others with bright blue cytoplasm and no granules, both generally having dense nuclei. Chromophobe mitoses (Pl. 3, fig. 4) are small and rather dense and consequently sometimes difficult to separate from pycnotic nuclei. The numbers given for dividing chromophobes in the Tables may on occasion therefore be too low. They are rather commoner in cells within the acidophil concentrations than peripherally where chromophobes are predominant. A curious association is very common in which a chromophobe is wrapped round an acidophil, either partially (Pl. 3, fig. 3) or apparently completely. Such appearances may have contributed to the theory of endocytogenesis (Franck, 1935, etc.). The basophils are relatively few, often isolated and distributed mainly laterally and ventrally. The full-sized examples are several times the area of an acidophil but there is a wide range both in size and granulation. They do not form a regular series as do the acidophils since degranulation and deep granulation are found in all sizes. There are also occasional slate-coloured basophils which may, like the others, possess one or more large vacuoles. Mitoses generally occur in medium-sized (Pl. 3, fig. 5) but occasionally in full-sized cells (Pl. 3, fig. 6). As with acidophils the degree of granulation does not seem to be a limiting factor to nuclear division and there is no dedifferentiation of the cell. Mitotic figures are frequently towards one side of the cell, as also are the nuclei, chromosomes are distinct and pycnotic nuclei rare. It is not uncommon for basophils to occur in which the granules are indistinct and the whole cell has a homogeneous appearance, with the nucleus to one side and difficult to make out. Possibly this is a form of degeneration giving rise eventually to the cell-sized masses of 'colloid' to be found in the lobe, especially during dioestrus. Occasionally exceptionally large basophils and acidophils are to be found.

The distal lobe is very well vascularized and the vessels come together along the anterior border, continuing by the ventral side of the hypophysial stalk into the brain. These vessels break up the tuberal lobe very extensively but despite the good blood supply there is a minimum of mitotic activity. In the small dull basophils, often vacuolated, which form the base of the lobe and intermingle with the distal lobe cells, no mitoses have been seen, but there is a single example in a cell on the base of the brain after six oestrone injections. One or more ciliated cysts were found in 20% of the glands, similar to those of the guinea-pig (Kirkman, 1937) except that they may occur anywhere in the distal lobe; they do not resemble the hypophysial cavity, which is without contents and has a different type of non-ciliated epithelium. Small lymphocyte cells (Pl. 3, fig. 8), with dense spherical nuclei slightly indented on one side and very scanty cytoplasm, are common throughout the lobe.

Changes during the oestrous cycle

The first and second periods of dioestrus (each about 24 hr.) have pituitaries showing very little mitotic activity (see Table 1 and Graph 1). The chromophobes, which are rarely entirely quiescent, show some activity in a few individuals but only isolated mitoses occur in other cell types. In the first period the predominating basophils are degranulated and small, in the second period there is increased granulation in some specimens but there is an overlap between the two groups. Small masses of homogeneous blue colloid are found here and in the next period more commonly than in the rest of the cycle.

In the third period of dioestrus (about 17 hr.) chromophils and proximal lobe cells, with the exception of one gland, show fairly consistent activity. Among the acidophils pycnotic nuclei are more common than mitoses. Chromophobe activity in this period and the next is more consistent rather than much more marked compared with the preceding. The basophils still show a wide staining range but there are considerably more in the heavily granulated condition than elsewhere in the oestrous cycle, in other words they appear to regranulate during dioestrus and reach their peak at its end. The proximal lobe mitoses show no tendency to localization in any particular part of the lobe.

In pro-oestrus (about 5 hr.) great mitotic activity was found in one animal and some in another, but the remainder were relatively inactive. In such cases there must remain some doubt as to whether the great activity of the one indicates an aberrant mouse or simply an animal in which activity has been fortuitously arrested towards its maximum. Owing to the short duration of this period there may be some overlap involved but in the mitotically active animals there is a change in the basophils. They show a decrease in the proportion of heavily granulated cells and a good deal of vacuolation, sometimes with one vacuole half the size of the cell or a series of vacuoles almost filling it. The loss of granulation continues in a very roughly progressive way until the end of oestrus. Vacuolation is quite common in later oestrus but occurs sporadically elsewhere. Division amongst the proximal lobe cells has largely passed, one mouse shows extensive vacuolation here.

uncertain. Pycnosis is prominent among acidophils and chromophobes. The remaining mouse shows no stimulatory effects at all, its pituitary is in a typically quiescent early dioestrous condition for which no explanation can be offered. In this and subsequent series there is occasionally vacuolation of the basophils and some colloid but never enough to be definitely abnormal.

Two and three injections produced a very marked contrast to the last. Mitoses in chromophil and proximal lobe cells are isolated and rare, and only one animal appears to have definitely stimulated chromophobes. Pycnotic nuclei in acidophils are only present in one specimen. These are so common after one injection that they must presumably have appeared and disappeared—if so they cannot have any great persistence.

Table 1. *Showing the numbers of mitoses in different cell types of the pituitary throughout the normal oestrous cycle and after one to six injections of oestrone. In each group the numbers for individual mice are given in the same sequence under the various cell types*

	Acidophils	Chromophobes	Basophils	Proximal lobe
Dioestrus I	0, 0, 0, 0, 0	1, 7, 1, 0, 0	0, 0, 0, 0, 0	0, 1, 1, 0, 0
Dioestrus II	0, 0, 0, 0, 0	4, 0, 3, 0, 0	1, 0, 0, 0, 0	0, 0, 0, 0, 0
Dioestrus III	3, 2, 3, 0, 0	2, 4, 3, 4, 1	0, 1, 2, 0, 0	3, 2, 3, 3, 0
Pro-oestrus	0, 8, 3, 0, 0	3, 14, 4, 5, 2	0, 3, 0, 1, 0	0, 1, 1, 0, 0
Oestrus I	0, 1, 0, 0, 0	1, 2, 2, 4, 3	0, 0, 0, 2, 0	0, 1, 0, 0, 0
Oestrus II	1, 0, 1, 1, 2	5, 8, 5, 6, 3	1, 0, 2, 0, 1	1, 0, 3, 0, 4
Oestrus III	2, 8, 3, 0, 4	5, 21, 13, 9, 19	1, 4, 3, 4, 2	3, 2, 5, 2, 4
Metoestrus	0, 0, 0, 0, 0	1, 2, 4, 6, 3	0, 0, 1, 0, 1	0, 1, 0, 0, 2
1 injection	10, 0, 15, 7, 26	12, 2, 20, 13, 23	5, 0, 6, 3, 9	2, 0, 9, 6, 3
2 injections	0, 0, 0, 0, 0	3, 2, 5, 1, 0	0, 0, 1, 0, 0	1, 0, 0, 0, 0
3 injections	0, 0, 0, 0, 0	3, 0, 2, 11, 2	0, 0, 0, 0, 1	0, 0, 1, 0, 0
4 injections	0, 0, 0, 2, 1	3, 7, 8, 6, 2	1, 2, 0, 0, 0	1, 0, 0, 0, 0
5 injections	0, 3, 0, 2, 0	3, 6, 1, 4, 7	1, 3, 0, 0, 2	0, 0, 0, 0, 1
6 injections	1, 3, 1, 0, 4	23, 11, 14, 7, 31	4, 1, 0, 2, 3	3, 2, 5, 0, 2

In the full and late oestrous periods (about 10 hr. each) the return of mitotic activity is more general and marked than before, reaching its climax in the second period. In contrast to the rat the basophils also are involved, although the number of mitoses in general is very much less (Hunt, 1942).. Degranulation of the basophils also reaches its maximum at the end of oestrus, in the acidophils no granulation changes have been detected but small changes would certainly be obscured by the number and normally very great staining range of the cells.

In metoestrus (about 7 hr.) there is a return to inactivity, though pycnosis still appears. The majority of the basophils remain degranulated or small.

Changes after oestrone injections

One injection has a most stimulating effect upon four pituitaries out of the five. All cell types are affected, although the relationship between their activities in any one gland is as usual somewhat

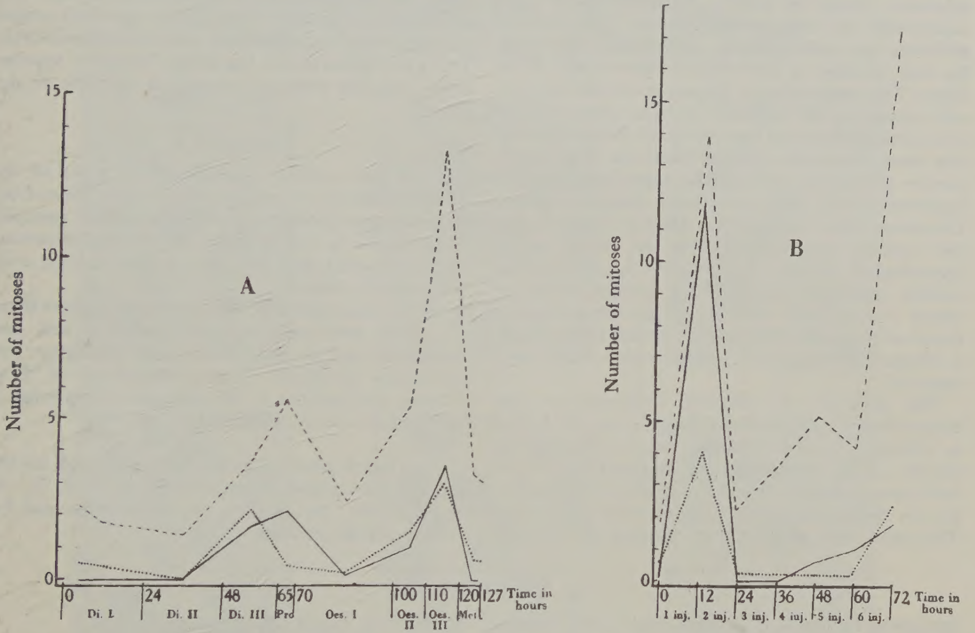
Four and five injections show a distinct but irregular activity in chromophils and one less distinct in chromophobes.

Six injections show an increase in chromophil mitoses which is more one of consistency than of numbers, in fact the smallness of their numbers in the last three injected series may indicate that even the very approximate regularity of the normal cycle is breaking down. The chromophobes show a very marked stimulation which if continued would produce an enlarged and chromophobic gland (Halpern & D'Amour, 1936). The increase in the proximal lobe mitoses is marked and fairly consistent. Granulation of the basophils in the injected mice shows only irregular differences after one to four injections, possibly because the injections started when it was at its lowest, after the last two injections however there are some glands showing a great lack of granulated cells. This is particularly striking if they are compared not with the previously injected mice but with normal mice at the end of dioestrus.

DISCUSSION

Mitotic activity during the oestrous cycle in the female mouse pituitary shows two maxima and two minima. There is a maximum just preceding the onset of oestrus, a drop in early oestrus, a recovery in full oestrus leading to a second higher maximum in late oestrus, and a final falling away through metoestrus to the relatively inert stages of the first and second periods of dioestrus. This conclusion might appear to be insufficiently supported owing to the small numbers of mitoses involved and their lack of consistency were it not for the evidence of the injected series. Here the differences are more accentuated, with great activity after one injection followed by a quiescent state after two and three injections, an increase in activity after four and five injections leading to a new maximum after six. Essentially the same problem is presented by both normal and injected groups, for in each there is the condition of a continued persistence, and perhaps increase, of oestrone in the blood accompanied by two discontinuous periods of mitotic activity. This break between the two maxima may be explained either by supposing that cells become susceptible to mitogenic stimulation in two waves in each oestrous cycle or that an outburst of cell division somehow induces a short period of inhibition. The latter appears more satisfactory since it would apply equally well to both normal and injected groups. The stimulation of cells becoming susceptible to division therefore could be supposed to begin in the normal cycle when oestrone from the ovary had reached a certain level in the blood, the resulting mitoses would be followed by a short inhibitory period and finally by the division of the remaining susceptible cells while the hormone content of the blood was still high. The second minimum would be the result less of a second inhibition than simply of a lack of oestrone. If inhibition does in fact occur, it would seem from the small amount of evidence available either that it affects the cell types of the pituitary rather differently or that a separate inhibitor must be supposed to originate from each cell type.

The variability of mitotic rate in the normal cycle may then be looked for in other factors. Two such unknowns which may be expected to vary with individual mice are the level of oestrone normally in the blood at any particular point in the cycle and



Graph 1. Graphs obtained by averaging the numbers of mitoses in the above tables for (A) the normal oestrous cycle, and (B) the oestrone injected series. In the normal series the points of the graph are entered above the middle of the appropriate periods. The injected series starts at the beginning of dioestrus and the points of the graph are entered above the twelve-hourly intervals at which the mice were killed. Acidophil mitoses, continuous line; chromophobe mitoses, broken line; proximal lobe mitoses, dotted line; basophils are omitted.

the susceptibility to division of the cells of any one organ. A further error on the experimental side may be introduced by the difference in the fixed colchicine-injected period ($9\frac{1}{2}$ hr.) and the varying lengths of the periods of oestrus, so that an optimum result cannot be ensured. An injection of hormone considerably in excess of the normal not only fixes the time of stimulation, producing comparable effects in each mouse, but can be expected to overcome small differences in cell susceptibility. The differences between individual mice still remain great however, as can be seen in the counts after one injection. Little is known of what makes cells susceptible to mitotic stimulation but clearly sufficient new cells must be produced to make up for any wastage in the normal functioning of an organ. The organization of the necessary cell division will depend on the internal conditions characteristic of a particular sex and species. Even if the stimulation were always due to 'sex' hormone the present pattern of activity can only be expected in female mammals with short successive oestrous cycles. Ovulation occurs during the interval between the two maxima but whether this, or other related physiological events, are related to the mitotic activity cannot be discussed on present evidence, which is concerned primarily with the mitogenic aspect of oestrone. The vaginal smear itself also is a reflexion of an effect of oestrone which has already occurred.

The presence of granules, indicative of the differentiated condition of the cell, is no hindrance to mitosis (see also Pomerat, 1941). The mitotic activity of the chromophobes suggests that these cells have important functions, of which a contribution to the chromophils is an obvious possibility. This gains some support from the lack of any great break in histological appearance between chromophobes and chromophils, the frequently isolated positions of basophils and the tendency for the chromophobe mitoses to be commoner amongst the acidophils. On the other hand the chromophils do to some degree at least replace themselves, and after castration Pomerat (1941) has shown that the multiplication of the basophils is by itself sufficient to cover the increase in these cells. Indeed until the extent of cell necrosis can be accurately measured the question of replacement is likely to remain open. As regards degranulation the number of the acidophils, the irregularity of their distribution and their normal staining range is so great that only major changes would be convincing and such do not occur. The granulation of the basophils, however, appears to be reduced with an increase of oestrone in the blood.

SUMMARY

1. Mitotic activity in the three cell types of the distal lobe and in the cells of the proximal lobe of the female mouse pituitary during the normal oestrous cycle occurs in two maxima, one in late dioestrus and pro-oestrus and the other in late oestrus, with intervening minima.

2. During a series of six oestrone injections there are again two maxima, after the first and last injections, and an intervening minimum after two and three injections. The relationships of these maxima and minima to the mitogenic properties of oestrone have been discussed.

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EXPLANATION OF PLATE 3

Fig. 1. Sagittal section of pituitary in situ. *N.l.* nervous lobe; *P.l.* proximal lobe; *D.l.* distal lobe; *St.* hypophysial stalk; *Br.* brain; *C.* floor of cranium. The hypophysial cavity lies between the proximal and distal lobes. × 50. Decalcified. Azan.

Fig. 2. Mitosis in acidophil. × 1000. Haemalum and eosin.

Fig. 3. Acidophil partially enveloped by chromophobe. × 1000. Haemalum and eosin.

Fig. 4. Mitosis in chromophobe. × 1000. Iron haemalum and Mallory.

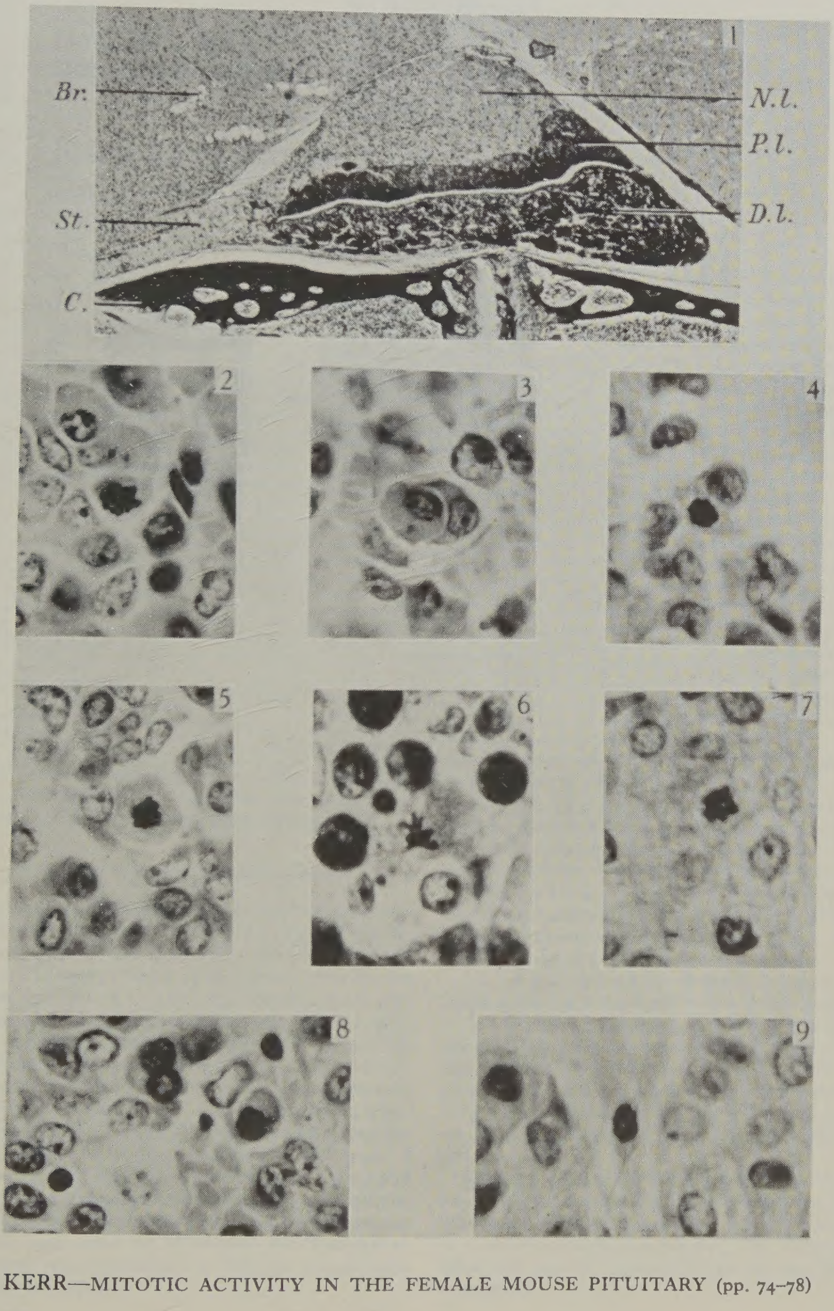
Fig. 5. Mitosis in medium-sized basophil. × 1000. Iron haemalum and Mallory.

Fig. 6. Mitosis in large basophil. The mitotic figure is in the lower left-hand corner of the cell, of which the granules are in focus. × 1000. Iron haemalum and Mallory.

Fig. 7. Mitosis in proximal lobe cell. × 1000. Haemalum and eosin.

Fig. 8. Pycnotic nucleus in acidophil. A binucleate acidophil can also be seen, and a lymphocyte cell lower left. × 1000. Haemalum and eosin.

Fig. 9. Mitosis in nervous lobe cell. An out-pushing of the nervous lobe from above is shown between two areas of the proximal lobe. × 1000. Haemalum and eosin.



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